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(54) Title: PFS28 FUSION PROTEINS

## (57) Abstract

This invention relates to an immunogenic composition capable of eliciting an immunogenic response directed to an epitope comprising an isolated Pfs28 and an isolated molecule comprising the epitope. The invention is also directed to methods of eliciting an immunogenic response directed to an epitope comprising administering an isolated Pfs28 and an isolated molecule comprising the epitope. This invention also relates to Pfs25-Pfs28 nucleic acids and fusion proteins. Cells, expression systems, and immunogenic compositions related to the nucleic acids and fusion proteins are provided. Methods of blocking transmission of malarial parasites using compositions of the invention are also provided.

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## **PFS28 FUSION PROTEINS**

### **CROSS REFERENCE TO RELATED APPLICATIONS**

The present application is a Continuation-In-Part application ("CIP") of U.S. Provisional application serial no. 60/027,390, filed September 30, 1996. This application is related to U.S. Patent No. 5,527,700 to Kaslow and Duffy, issued June 18, 1996 and to U.S. Patent No. 5,217,898 to Kaslow and Barr issued Jun 8, 1993. Each of the aforementioned documents is explicitly incorporated herein by reference in their entirety and for all purposes.

### **FIELD OF THE INVENTION**

This invention relates to immunogenic compositions and methods for eliciting an immunogenic response directed to a desired epitope. In particular, the invention relates to use of compositions comprising isolated an Pfs28 polypeptide and an isolated molecule comprising the epitope.

### **BACKGROUND OF THE INVENTION**

Immunization against desired antigens associated with infectious diseases, cancers and the like is an important approach to the treatment and prevention of disease. In some instances, for example, subunit vaccines, the antigen may not be complex enough to generate an appropriate immune response. Thus, the immunogenicity of antigens is sometimes increased by administering the antigen with a carrier protein to create a conjugate vaccine. A primary purpose for use of carriers is to achieve more durable humoral or cell-mediated immunity of a high level by employing lower levels of an antigen with fewer numbers of doses than could otherwise be achieved.

One particularly useful application of immunization is in the control of malaria. Malaria continues to exact a heavy toll on humans. Between 200 million to 400 million people are infected by *Plasmodium falciparum*, the deadliest of the malarial protozoans, each year. One to four million of these people die. Approximately 25 percent

of all deaths of children in rural Africa between the ages of one and four years are caused by malaria.

The life cycle of the malaria parasite is complex. Infection in man begins when young malarial parasites or "sporozoites" are injected into the bloodstream of a human by a mosquito. After injection, the parasite localizes in liver cells. Approximately one week after infection, the parasites, now formed "merozoites" are released into the bloodstream to begin the erythrocytic phase. Each parasite enters a red blood cell in order to grow and develop. When the merozoite matures in the red blood cell, it is known as a "trophozoite" and, when fully developed, as a "schizont". A schizont is the stage when nuclear division occurs to form individual merozoites, which are released to invade other red cells. After several schizogonic cycles, some parasites, instead of becoming schizonts through asexual reproduction, develop into large uninucleate parasites. These parasites undergo sexual development.

Sexual development of the malaria parasites involves the female "macrogametocyte" and the male "microgametocyte". These gametocytes do not undergo any further development in man. Upon ingestion of the gametocytes into the mosquito, the complicated sexual cycle begins in the midgut of the mosquito. The red blood cells disintegrate in the midgut of the mosquito after 10 to 20 minutes. The microgametocyte continues to develop through exflagellation and releases 8 highly flagellated microgametes. Fertilization occurs with the fusion of a microgamete and a macrogamete. The fertilized parasite, which is known as a zygote, then develops into an ookinete. The ookinete penetrates the midgut wall of the mosquito and develops into an oocyst, within which many small sporozoites form. When the oocyst ruptures, the sporozoites migrate to the salivary gland of the mosquito via the hemolymph. Once in the saliva of the mosquito, the parasite can be injected into a host, and the life cycle repeated.

Malaria vaccines are needed against different stages in the parasite's life cycle, including the sporozoite, asexual erythrocyte, and sexual stages. Each vaccine against a particular life cycle stage increases the opportunity to control malaria in the many diverse settings in which the disease occurs. For example, sporozoite vaccines fight infection immediately after injection of the parasite into the host by the mosquito.

Vaccines of this type have been tested in humans. Asexual erythrocytic stage vaccines are

useful in reducing the severity of the disease. Multiple candidate antigens for this stage have been cloned and tested in animals and in humans.

However, as drug-resistant parasite strains render chemoprophylaxis increasingly ineffective, a great need exists for a transmission-blocking vaccine. Such a vaccine would block the portion of the parasite's life cycle that takes place in the mosquito or other arthropod vector, preventing even the initial infection of humans. U.S. Patent No. 5,527,700 to Kaslow and Duffy, issued June 18, 1996 and U.S. Patent No. 5,217,898 to Kaslow and Barr issued Jun 8, 1993 provide two highly useful transmission blocking antigens (pfs 25 and pfs 28) useful as transmission blocking vaccines. Compositions which have antigenic transmission blocking properties similar to multiple antigens (*e.g.*, both pfs 25 and pfs 28), which have increased antigenicity, and which provide for simplified manufacturing are desirable. The present invention solves these and other problems.

The development of stable vaccine compositions that can elicit both humoral and cellular responses against various pathogens, such as *Plasmodium falciparum*, would be desirable. The present invention addresses these and other needs.

### SUMMARY OF THE INVENTION

Pfs28 as an immunogenic carrier is provided for by the invention. It was surprisingly discovered that Pfs28, administered with a second composition, provides a superior antigenic response to the second composition. Thus, the invention relates to an immunogenic composition capable of eliciting an immunogenic response directed to an epitope comprising an isolated Pfs28 and an isolated molecule comprising the epitope. The invention is also directed to methods of eliciting an immunogenic response directed to an epitope using the compositions of the invention. The Pfs28 and the second molecule can be chemically linked or joined together as recombinant fusion proteins.

In one embodiment, the Pfs28-containing fusion protein is a Pfs25-Pfs28 protein. It was surprisingly discovered that the sexual stage malarial proteins Pfs25 and Pfs28, in the form of a Pfs25-Pfs28 fusion protein, generate transmission-blocking antibodies against both Pfs25 and Pfs28. These fusion proteins have enhanced antigenic properties, as compared to use of either alone as an immunogen. These fusion proteins also provide for simplified manufacturing of Pfs25-Pfs28 antigens.

In one class of embodiments, the Pfs25-Pfs28 fusion protein includes an N terminal Pfs25 domain and a C terminal Pfs28 domain. It was surprisingly discovered that this arrangement of Pfs25 and Pfs28 in a fusion protein provides superior antigenic and transmission blocking properties for the fusion protein. In one preferred embodiment, the C terminal Pfs28 domain includes the carboxyl terminal region of Pfs28. Exemplary fusion proteins are provided in the examples set forth herein, and conservative modifications thereof.

Typically, the Pfs28 fusion proteins of the invention include a flexible linker separating the Pfs28 domains and the antigen. In the case of Pfs28-Pfs25 fusions, an exemplary flexible linker is the amino acid sequence GGGPGGG.

In one embodiment, the fusion protein is produced recombinantly. In one particularly preferred embodiment, a nucleic acid encoding a fusion protein of the invention is optimized for expression in yeast, thereby facilitating recombinant expression and manufacturing of the fusion protein. In other embodiments, the different domains of the immunogenic composition are joined, or linked, together by chemical means. In further embodiments, the domains of the immunogenic compositions are derived from natural sources.

The Pfs28 fusion proteins, when administered to a mammal, elicit the production of at least two classes of antibodies: antibodies which specifically bind to the antigen and antibodies which specifically bind to Pfs28. In the case of Pfs25-Pfs28 fusions, the administration of the fusion proteins of the invention elicit a transmission blocking immune response. Immunological enhancers and pharmaceutically acceptable carriers are optionally added to the fusion protein to enhance the immunogenicity of the fusion protein and to facilitate delivery of the fusion protein to a mammal. For example, adjuvants such as alum are optionally added.

Immunogenic compositions comprising the fusion proteins of the invention elicit transmission blocking antibodies in a variety of mammals, including humans and other primates, and mice and other rodents.

Nucleic acids encoding the fusion proteins of the invention are also provided. Exemplar nucleic acids are described in the sequence listings herein, and by reference to the polypeptide sequences herein, which are optionally used to provide

corresponding coding nucleic acids via the genetic code. In a preferred embodiment, the nucleic acids of the invention comprise yeast preferred codons which enhance translation of the nucleic acid in yeast. Expression of the nucleic acid in yeast optionally and preferably provides secreted fusion protein from a yeast culture at a level in excess of 5 mg/L.

The nucleic acids of the invention optionally comprise a pharmaceutical excipient, and are optionally injected into a host to induce transmission blocking antibodies against encoded polypeptides.

Cells expressing the nucleic acids and polypeptide of the invention are a feature of the invention. For example, recombinant cells such as yeast cells can be used to express the Pfs28 fusion protein of the invention.

The invention provides methods of inducing a transmission blocking antibody in a mammal. In the methods, the Pfs25-Pfs28 fusion protein, or a nucleic acid encoding the fusion protein is administered to a mammal, which produces transmission blocking antigens. Administration is typically performed intramuscularly, intradermally, or subcutaneously. An adjuvant such as alum is optionally administered with the fusion protein or nucleic acid.

A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification, the figures and claims. All publications, patents and patent applications cited herein are hereby expressly incorporated by reference for all purposes.

## DEFINITIONS

For purposes of the present invention, the following terms are specifically defined below.

An "immunogen" refers to a compound or composition which is "immunogenic," *i.e.*, capable of eliciting, augmenting or boosting a cellular and/or humoral immune response, either alone or in combination or linked or fused to another substance. An immunogenenic composition can be any biomolecule, such as carbohydrates, polypeptides, lipids, nucleic acids, and the like. If the immunogen is a peptide it is typically at least about 5 to about 500 amino acids in length, usually 20 amino acids to about 250 amino acids in length, or from about 25 to about 50 amino acids in length. The

immunogen can comprise a "carrier" polypeptide and a hapten, *e.g.*, a fusion protein or a carrier polypeptide fused or linked (chemically or otherwise) to another composition (described below). The immunogen can be recombinantly expressed in an immunization vector, which can be simply naked DNA comprising the immunogen's coding sequence operably linked to a promoter. The immunogen includes antigenic determinants, or epitopes (described below), to which antibodies or TCRs bind, which are typically 3 to 10 amino acids in length.

The term "Pfs28 polypeptide" refers to a 28kD protein expressed on the surface *Plasmodium falciparum* ookinetes and variations thereof, as defined below. The term encompasses native, full length proteins as well as recombinantly produced or modified proteins or fragments thereof. It also includes immunologically active fragments of these proteins. A Pfs28 polypeptide of the invention contains at least about 10 amino acids, usually at least about 50 and more often 100 to about 220 amino acids. The Pfs28 polypeptide can be an immunogen or an immunogenic carrier, or it can be a hapten, *i.e.*, a Pfs28 fragment/epitope that must be linked to a carrier to generate an epitope-specific immune response. In the expression of recombinantly produced or transgenically produced Pfs28 polypeptides, one of skill will recognize that the inserted polynucleotide sequence (the Pfs28 polynucleotide) need not be identical to a native sequence and may be "substantially identical" to a sequence of the gene from which it was derived or encode a protein which is "a conservatively modified variation" of the native sequence. As explained below, these variants are specifically covered by the term Pfs28 polypeptide or Pfs28 polynucleotide. In the case where the inserted Pfs28 polynucleotide sequence is transcribed and translated to produce a Pfs28 polypeptide, one of skill will recognize that because of codon degeneracy a number of polynucleotide sequences will encode the same polypeptide. These variants are specifically covered by the above terms. In addition, the term "polynucleotide sequence from a Pfs28 gene" specifically includes those sequences substantially identical or "a conservatively modified variation" (determined as described below) with a native Pfs28 sequence. Thus, in the case of the Pfs28 gene disclosed here, the above term includes variant polynucleotide sequences which have substantial identity or "conservatively modified variations" with the sequences disclosed herein and which encode proteins capable of inducing a transmission blocking immune response.



A "Pfs28 fusion protein" refers to a composition comprising at least one Pfs28 polypeptide domain which is associated with a second domain. The second domain can be a polypeptide, peptide, polysaccharide, or the like. The "fusion" can be an association generated by a chemical linking or by a charge (electrostatic attraction, *i.e.*, salt bridges, H-bonding, etc.) interaction. If the polypeptides are recombinant, the "fusion protein" can be translated from a common message. Alternatively, the compositions of the domains can be linked by any chemical or electrostatic means.

A "Pfs25-Pfs28 fusion protein" refers to a polypeptide comprising at least two domains, with polypeptide subsequences derived from both Pfs25 and Pfs28. The fusion protein typically includes about 10 contiguous amino acids or more, preferably 15 contiguous amino acids or more, often 20 contiguous amino acids or more and generally 25 contiguous amino acids or more from both Pfs 25 and Pfs28. The fusion protein optionally comprises additional subsequences which are not derived from Pfs25 or Pfs 28, such as a flexible linker region separating the Pfs25 and Pfs28 subsequences.

An "N terminal" or "C terminal" domain in reference to a specified protein refers to a polypeptide subsequence derived from the N terminal or C terminal half of the indicated protein. For example, an N terminal Pfs25 protein domain refers to a polypeptide subsequence derived from the N terminal half of the Pfs25 protein. Similarly, a C terminal Pfs28 protein domain refers to a polypeptide subsequence derived from the C terminal half of the Pfs28 protein. The subsequence is from about 10 amino acids in length up to the entire specified half protein.

The terms "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state.

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence optionally includes or represents the complementary sequence thereof.

The term "subsequence" in the context of a particular nucleic acid sequence

or polypeptide refers to a region of the nucleic acid or polypeptide equal to or smaller than the specified nucleic acid or polypeptide.

A "recombinant nucleic acid" comprises or is encoded by one or more nucleic acids that are derived from a nucleic acid which was artificially constructed. For example, the nucleic acid can comprise or be encoded by a cloned nucleic acid formed by joining heterologous nucleic acids as taught, *e.g.*, in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* volume 152 Academic Press, Inc., San Diego, CA (Berger) and in Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3 (Sambrook). Alternatively, the nucleic acid can be synthesized chemically. The term "recombinant" when used with reference to a cell indicates that the cell replicates or expresses a nucleic acid, or expresses a peptide or protein encoded by a nucleic acid whose origin is exogenous to the cell. Recombinant cells can express genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also express genes found in the native form of the cell wherein the genes are re-introduced into the cell or a progenitor of the cell by artificial means.

Two single-stranded nucleic acids "hybridize" when they form a double-stranded duplex. The region of double-strandedness can include the full-length of one or both of the single-stranded nucleic acids, or all of one single stranded nucleic acid and a subsequence of the other single stranded nucleic acid, or the region of double-strandedness can include a subsequence of each nucleic acid. An overview to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes* part 1 chapter 2 "overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York.

The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using the programs described above using standard parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like.

Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 70%, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%. Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions.

The term "stringent conditions" in the context of nucleic acid hybridization refers to conditions which are sequence dependent and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993), *id.* Generally, stringent conditions are selected to be about 5° C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength and pH. The  $T_m$  is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Highly stringent conditions are selected to be equal to the  $T_m$  point for a particular probe. Nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

The term "identical" in the context of two nucleic acid or polypeptide sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence. When percentage of sequence identity is used in reference to proteins or peptides it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acids residues are substituted for other amino acid residues with similar chemical properties (*e.g.* charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, *e.g.*, according to known algorithm. *See, e.g.*, Meyers and

Miller, *Computer Applic. Biol. Sci.*, 4: 11-17 (1988); Smith and Waterman (1981) *Adv. Appl. Math.* 2: 482; Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443; Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444; Higgins and Sharp (1988) *Gene*, 73: 237-244 and Higgins and Sharp (1989) *CABIOS* 5: 151-153; Corpet, *et al.* (1988) *Nucleic Acids Research* 16, 10881-90; Huang, *et al.* (1992) *Computer Applications in the Biosciences* 8, 155-65, and Pearson, *et al.* (1994) *Methods in Molecular Biology* 24, 307-31. Alignment is also often performed by inspection and manual alignment.

"Conservatively modified variations" of a particular nucleic acid sequence refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of "conservatively modified variations." Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

The term "conservatively modified variations" refers to individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence, where the alterations result in the substitution of an amino acid with a chemically similar amino acid; and the alterations, deletions or additions do not alter the structure, function and/or immunogenicity of the sequence. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following

six groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

A "vector" is a composition which can transduce, transfect, transform or infect a cell, thereby causing the cell to replicate or express nucleic acids and/or proteins other than those native to the cell, or in a manner not native to the cell. A cell is "transduced" by a nucleic acid when the nucleic acid is translocated into the cell from the extracellular environment. Any method of transferring a nucleic acid into the cell may be used; the term, unless otherwise indicated, does not imply any particular method of delivering a nucleic acid into a cell, nor that any particular cell type is the subject of transduction. A cell is "transformed" by a nucleic acid when the nucleic acid is transduced into the cell and stably replicated. A vector includes a nucleic acid (ordinarily RNA or DNA) to be expressed by the cell. This nucleic acid is optionally referred to as a "vector nucleic acid." A vector optionally includes materials to aid in achieving entry of the nucleic acid into the cell, such as a viral particle, liposome, protein coating or the like. A "cell transduction vector" is a vector which encodes a nucleic acid which is expressed in a cell once the nucleic acid is transduced into the cell.

A "promoter" is an array of nucleic acid control sequences which direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive" promoter is a promoter which is active under most environmental and developmental conditions. An "inducible" promoter is a promoter which is under environmental or developmental regulation. A "tissue specific" promoter is active in certain tissue types of an organism, but not in other tissue types from the same organism. The term "operably linked" refers to a functional linkage between a nucleic acid

expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

A "transmission blocking antibody" is an antibody which inhibits the growth or replication of a malarial parasite during the sexual stage of parasite development in the mosquito gut. The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. An exemplar immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain ( $V_L$ ) and variable heavy chain ( $V_H$ ) refer to these light and heavy chains respectively. Antibodies exist *e.g.*, as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce  $F(ab)'_2$ , a dimer of Fab which itself is a light chain joined to  $V_H-C_H1$  by a disulfide bond. The  $F(ab)'_2$  may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the  $F(ab)'_2$  dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see, Fundamental Immunology*, Third Edition, W.E. Paul, ed., Raven Press, N.Y. (1993), which is incorporated herein by reference, for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA methodologies. Immunoglobulins

generated using recombinant expression libraries are also antibodies for purposes of this invention.

An "immunogenic composition" is a composition which elicits the production of antibodies or a cell-mediated immune response when administered to a mammal.

An "immunological carrier" or "carrier" in the immunological context (as opposed to a carrier which is a nonactive composition for the purpose of formulating, storing or carrying a pharmaceutical) is a compound which, when provided in combination with a second composition (*e.g.*, protein, peptide, polysaccharide or the like) boosts or augments the cellular or humoral response to the composition. The two components may be linked or unlinked/ Any physiologic mechanism can be involved in this augmentation or boosting of the immune response. An immunological carrier is typically a polypeptide linked or fused to a second composition of interest comprising a protein, peptide or polysaccharide, where the carrier stimulates a cellular (T cell mediated) immune response that boosts or augments the humoral (B cell mediated, antibody-generating) immune response to the composition of interest. These second compound can be a "hapten," which is typically defined as a compound of low molecular weight that is not immunogenic by itself, but that, when coupled to carrier molecules, can elicit antibodies directed to epitopes on the hapten. For example, the lack of an adequate immune response to the major polysaccharide of the *Haemophilus influenzae* type b capsule (PRP) in very young infants can be overcome by conjugating PRP to a T-cell dependent carrier protein (see Zepp (1997) *Eur. J. Pediatr.* 156:18-24). Alternatively, a peptide can be linked to a carrier simply to facilitate manipulation of the peptide in the generation of the immune response (see, for example, Rondard (1997) *Biochemistry* 36:8962-8968).

An "epitope" refers to an antigenic determinant or antigen site that interacts with an antibody or a T cell receptor (TCR). An "antigen" is a molecule or composition that induces the production of an immune response. An antibody or TCR binds to a specific conformational (possibly charge-dependent) domain of the antigen, called the "antigenic determinant" or "epitope" (TCRs bind the epitope in association with a third molecule, a major histocompatibility complex (MHC) protein).

## DESCRIPTION OF THE PREFERRED EMBODIMENT

This invention relates to an immunogenic composition capable of eliciting an immunogenic response directed to a desired epitope. The compositions of the invention comprise an isolated Pfs28 polypeptide and an isolated molecule comprising the epitope. The invention is also directed to methods of eliciting an immunogenic response directed to an epitope comprising administering the compositions of the invention to an individual.

Epitopes from a variety of pathogens and cancer antigens can be used in the invention. Examples of antigens useful in the invention include prostate specific antigen (PSA), hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, melanoma antigens (*e.g.*, MAGE-1), colon cancer antigens (*e.g.*, CEA), breast cancer antigens (*e.g.*, HER-2) human immunodeficiency virus (HIV) antigens, herpes virus antigens, hepatitis (*e.g.*, A, B, or C) tyrosinase, trp-1, malarial antigens, or human papilloma virus (HPV) antigens.

The present invention also relates to novel compositions and methods for blocking transmission of parasites responsible for malaria utilizing Pfs25-Pfs28 fusion proteins and nucleic acids. The invention provides agents capable of inhibiting the life cycle of the material parasite in the mosquito midgut.

The fusion proteins of the invention (optionally used with an adjuvant such as alum) can be used to block transmission of a number of parasites associated with malaria. Examples of parasites whose transmission is blocked by the materials and compositions of the invention include the causative parasites for malaria. Four species of the genus *Plasmodium* infect humans, *P. vivax*, *P. ovale*, *P. malariae*, and *P. falciparum*. *P. falciparum* is the most prevalent cause of malaria in humans. Other *Plasmodium* species infect other animals. For instance, *P. gallinaceum* is responsible for avian malaria.

The use of Pfs25 as a transmission blocking antigen is described, *e.g.*, in U.S. Patent 5,217,898 to Kaslow and Barr. The use of Pfs28 as a transmission blocking antigen is described, *e.g.*, in U.S. Patent 5,527,700 to Kaslow and Duffy.

The Pfs25-Pfs28 fusion proteins of the invention have several surprising properties. First, as described in the Examples below, the fusion protein is more efficient in producing transmission blocking antibodies, *e.g.*, in mice, than Pfs25 or Pfs28 alone. This is true despite the fact that it was discovered that a mixed dose of Pfs25 and Pfs28 did



not induce a higher level of transmission blocking antibody activity than either Pfs25 or Pfs28 alone. Second, less fusion protein is required as an immunogen than either Pfs25 or Pfs28 alone. Third, titres of transmission blocking antibodies remains high for a longer period of time when the antigen is a Pfs25-Pfs28 fusion protein than either Pfs25 or Pfs 28 alone. Fourth, the high immunogenicity of the fusion protein is surprising because it is often the case that the domains of a fusion protein interact to prevent presentation of one or more domains of the fusion protein to the immune system, thereby failing to elicit antibodies which recognize the domains of the fusion protein. For Pfs25-Pfs28 fusion proteins, antibodies which recognize both Pfs 25 and Pfs28 were elicited. Fifth, Pfs25 and Pfs28 are cysteine rich, which would have been expected to make the possibility of incorrect folding of the fusion protein to be high, resulting in a failure to elicit transmission blocking antibodies to Pfs 25 and/or Pfs28. This was determined not to occur. Sixth, it is often the case with a fusion protein that one domain of the protein will be immunologically dominant. This did not occur with the Pfs25-Pfs28 fusion proteins of the invention, as the fusion proteins elicit transmission blocking antibodies which recognize Pfs25 as well as transmission blocking antibodies which recognize Pfs28. Finally, in a preferred aspect, the present invention overcomes any potential fusion protein production problems by providing a nucleic acid with yeast preferred codons for encoding and expressing the fusion protein in yeast.

### Pfs28 Polypeptides

In one embodiment, the invention provides for immunogenic compositions including Pfs28 and fragments thereof. A gene encoding the protein is provided as SEQ ID NO:1. The encoded protein is SEQ ID NO:2. These Pfs28 proteins are useful for inducing an immune response when the proteins are injected into a human or other host animal.

In another embodiment, the immunogenic composition, comprising an isolated Pfs28 and an isolated molecule comprising the epitope, is capable of eliciting or augmenting an immunogenic response directed to the epitope. The Pfs28 can act as a immunological "carrier" to boost, augment or increase the cellular or humoral response to the epitope.

The antibodies or T cells that arise from a Pfs28, Pfs 25 or Pfs28-Pfs25 generated immune response block transmission of the parasite malaria by interfering with the portion of the parasite's life cycle that occurs in the mosquito. For example, purified polypeptides having an amino acid sequence substantially identical to a subsequence of Pfs28 may be used.

The Pfs28 proteins of the invention may be purified from parasites isolated from infected host organisms. Methods for purifying desired proteins are well known in the art and are not presented in detail here. For a review of standard techniques see, *Methods in Enzymology*, "Guide to Protein Purification", M. Deutscher, ed. Vol. 182 (1990), which is incorporated herein by reference. For instance, Pfs28, or its homologous polypeptides can be purified using affinity chromatography, SDS-PAGE, and the like.

#### Pfs25-Pfs28 Fusion Proteins

The present invention includes immunogenic polypeptides which comprise polypeptide subsequences derived from both Pfs28 and Pfs25. These polypeptides are useful for inducing an immune response when the fusion protein is injected into a human, mouse or other host animal. The antibodies that arise from the immune response block transmission of the malarial parasite by interfering with the portion of the parasite's life cycle that occurs in the mosquito.

The fusion proteins typically include an immunogenic domain from Pfs25 and an immunogenic domain from Pfs28. The immunogenic domains are polypeptide subsequences which are sufficient to elicit an antibody response against the domain when administered to a mammal (*e.g.*, a mouse). Preferably, the immunogenic domain elicits the production of an antibody which recognizes the corresponding full length protein. For example, if the immunogenic domain is a Pfs25 subsequence, the domain preferably elicits the production of an antibody which specifically binds to Pfs25. Similarly, if the immunogenic domain is a Pfs28 subsequence, the domain preferably elicits the production of an antibody which specifically binds to Pfs28.

To elicit the production of an antibody, the immunogenic domain is typically at least about 3- 10 amino acids in length, because the protein recognition site on an antibody typically recognizes an amino acid of about 3-10 amino acids in length. More

antibody typically recognizes an amino acid of about 3-10 amino acids in length. More often, the immunogenic domain is longer than 10 amino acids, and the domain optionally includes the full length sequence of the corresponding protein (*i.e.*, in one embodiment, the Pfs25-Pfs28 fusion protein comprises the complete sequence of both Pfs25 and Pfs28). Ordinarily, only a fraction of the full length protein is included. In one embodiment, about 10% of the full length antigen is included in the fusion protein. In another embodiment, about 20% of the full length antigen is included in the fusion protein. In yet another embodiment, about 30% of the full length protein is included. In still another embodiment, about 40% of the full length protein is included in the fusion protein. Optionally, as much as about 50% of the full length protein is included in the fusion protein. Occasionally, as much as about 60% of the full length protein is included in the fusion protein. In some embodiments, as much as about 70% of the full length protein is included in the fusion protein. In one class of embodiments, as much as about 80% of the full length protein is included in the fusion protein. As much as about 90% of the full length full length is optionally included in the fusion protein. As already mentioned, the entire full length full length protein is optionally incorporated into the fusion protein.

Similarly, in one embodiment, about 10% of the full length Pfs28 polypeptide is included in the fusion protein. In another embodiment, about 20% of the full length Pfs28 polypeptide is included in the fusion protein. In yet another embodiment, about 30% of the full length protein is included. In still another embodiment, about 40% of the full length Pfs28 polypeptide is included in the fusion protein. Optionally, as much as about 50% of the full length Pfs28 is included in the fusion protein. Occasionally, as much as about 60% of the full length Pfs28 is included in the fusion protein. In some embodiments, as much as about 70% of the full length Pfs28 is included in the fusion protein. In one class of embodiments, as much as about 80% of the full length Pfs28 is included in the fusion protein. As much as about 90% of the full length Pfs28 is optionally included in the fusion protein. As already mentioned, the entire full length Pfs28 protein is optionally incorporated into the fusion protein.

The portion of the antigen or the Pfs28 polypeptide from which the immunogenic domain is selected is optionally optimized for maximum immunogenicity. For example, it is found that the carboxyl terminal portion of Pfs28 is preferably included

for the induction of transmission blocking vaccines using Pfs25. The examples section below describes certain preferred embodiments. Other preferred embodiments include those derived from the particular fusion proteins described in the examples below, in which about 10-20 amino acids are deleted or added to the particular Pfs25 or Pfs28 subsequences described. The added or deleted amino acids are added or deleted by reference to the corresponding full length sequence, *e.g.*, where the subsequence is derived from Pfs25, a 10-20 amino acid sequence derived from Pfs25 is optionally added to either end of the subsequence.

The fusion proteins optionally includes additional features such as a flexible linker between antigen and the Pfs 28 domain which facilitates the independent folding of the proteins. Preferred flexible linkers are amino acid subsequences which are synthesized as part of a recombinant fusion protein. In one embodiment, the flexible linker is an amino acid subsequence comprising a proline such as Gly<sub>3</sub>-Pro-Gly<sub>3</sub>. In other embodiments, a chemical linker is used to connect synthetically or recombinantly produced Pfs25 and Pfs28 subsequences. Such flexible linkers are known to persons of skill in the art. For example, poly(ethylene glycol) linkers are available from Shearwater Polymers, Inc. Huntsville, Alabama. These linkers optionally have amide linkages, sulfhydryl linkages, or heterofunctional linkages.

In addition to flexible linkers, the fusion proteins optionally include polypeptide subsequences from proteins which are unrelated to Pfs25 or Pfs28, *e.g.*, a sequence with affinity to a known antibody to facilitate affinity purification, or the like.

Included among the polypeptides of the present invention are fusion proteins that have subsequences which are homologous to Pfs28 or Pfs25. Such homologs, also referred to as Pfs28 or Pfs25 polypeptides, respectively, include variants of the native proteins constructed by *in vitro* techniques, and proteins from parasites related to *P. falciparum*. For example, one skilled in the art will appreciate that for certain uses it is advantageous to produce a Pfs28 or Pfs25 polypeptide subsequence that is lacking a structural characteristic; for example, one may remove a transmembrane domain to obtain a polypeptide that is more soluble in aqueous solution.

One of skill will appreciate that many conservative variations of the fusion proteins and nucleic acid which encode the fusion proteins yield essentially identical

products. For example, due to the degeneracy of the genetic code, "silent substitutions" (*i.e.*, substitutions of a nucleic acid sequence which do not result in an alteration in an encoded polypeptide) are an implied feature of every nucleic acid sequence which encodes an amino acid. As described herein, sequences are preferably optimized for expression in a particular host cell used to produce the fusion protein (*e.g.*, yeast). Similarly, "conservative amino acid substitutions," in one or a few amino acids in an amino acid sequence are substituted with different amino acids with highly similar properties (*see*, the definitions section, *supra*), are also readily identified as being highly similar to a particular amino acid sequence, or to a particular nucleic acid sequence which encodes an amino acid. Such conservatively substituted variations of any particular sequence are a feature of the present invention.

One of skill will recognize many ways of generating alterations in a given nucleic acid sequence, which optionally provides alterations to an encoded protein. Such well-known methods include site-directed mutagenesis, PCR amplification using degenerate oligonucleotides, exposure of cells containing the nucleic acid to mutagenic agents or radiation, chemical synthesis of a desired oligonucleotide (*e.g.*, in conjunction with ligation and/or cloning to generate large nucleic acids) and other well-known techniques. *See*, Gilman and Smith (1979) *Gene* 8:81-97; Roberts *et al.* (1987) *Nature* 328:731-734 and Sambrook, Innis, Ausbel, Berger, Needham VanDevanter and Mullis (*below*).

Most commonly, amino acid sequences are altered by altering the corresponding nucleic acid sequence and expressing the polypeptide. However, polypeptide sequences are also optionally generated synthetically on commercially available peptide synthesizers to produce any desired polypeptide (*see*, Merrifield, and Stewart and Young, *supra*).

One can select a desired nucleic acid or polypeptide of the invention based upon the sequences and constructs provided and upon knowledge in the art regarding malaria generally. The life-cycle, genomic organization, developmental regulation and associated molecular biology of malaria strains have been the focus of research since the advent of molecular biology.

Moreover, general knowledge regarding the nature of proteins and nucleic acids allows one of skill to select appropriate sequences with activity similar or equivalent

herein describes exemplar conservative amino acid substitutions.

Finally, most modifications to nucleic acids and polypeptides are evaluated by routine screening techniques in suitable assays for the desired characteristic. For instance, changes in the immunological character of a polypeptide can be detected by an appropriate immunological assay. Modifications of other properties such as nucleic acid hybridization to a target nucleic acid, redox or thermal stability of a protein, hydrophobicity, susceptibility to proteolysis, or the tendency to aggregate are all assayed according to standard techniques.

### Pfs28 Fusion Nucleic Acids

Another aspect of the present invention relates to the cloning and recombinant expression of Pfs28 fusion proteins. The recombinantly expressed proteins are used in a number of ways. For instance, they can be used as immunogenic compositions, transmission-blocking vaccines, or to raise antibodies. The antibodies are useful as transmission blocking agents, and as immunological reagents for the identification of malarial parasites in biological samples such as insects or blood. In addition, cloned nucleic acids encoding the polypeptides can be used as probes to identify homologous polypeptides in other species, or to identify malarial nucleic acids in biological samples.

Thus, the invention uses techniques from the field of recombinant molecular biology. Indeed, given the strategy for making nucleic acids of the present invention which encode Pfs28 fusion proteins, one of skill can construct a variety of clones containing functionally equivalent nucleic acids. Cloning methodologies to accomplish these ends, and sequencing methods to verify the sequence of nucleic acids are well known in the art. Examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Publish., Cold Spring Harbor, NY 2nd ed. (1989) (Sambrook); and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1995 Supplement) (Ausubel). Product information from

Wiley & Sons, Inc., (1995 Supplement) (Ausubel). Product information from manufacturers of biological reagents and experimental equipment also provide information useful in known biological methods. Such manufacturers include the SIGMA chemical company (Saint Louis, MO), R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen, San Diego, CA, and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill.

The nucleic acids sequenced by this invention, whether RNA, cDNA, genomic DNA, or a hybrid of the various combinations, are isolated from biological sources, or synthesized *in vitro*. The nucleic acids of the invention are present in transformed or transfected whole cells, in transformed or transfected cell lysates, or in a partially purified or substantially pure form.

*In vitro* amplification techniques suitable for amplifying sequences to provide a nucleic acid or for subsequent analysis, sequencing or subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Q $\beta$ -replicase amplification and other RNA polymerase mediated techniques (*e.g.*, NASBA) are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.*, (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis *et al.* eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3, 81-94; (Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86, 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell *et al.* (1989) *J. Clin. Chem* 35, 1826; Landegren *et al.*, (1988) *Science* 241, 1077-1080; Van Brunt (1990) *Biotechnology* 8, 291-294; Wu and Wallace, (1989) *Gene* 4, 560; Barringer *et al.* (1990) *Gene* 89, 117, and Sooknanan and Malek (1995) *Biotechnology* 13: 563-564. Improved methods of cloning *in vitro* amplified nucleic acids are described in Wallace *et al.*, U.S. Pat. No. 5,426,039. Improved methods of amplifying large nucleic acids are summarized in Cheng *et al.* (1994) *Nature* 369: 684-685

and the references therein. One of skill will appreciate that essentially any RNA can be converted into a double stranded DNA suitable for restriction digestion, PCR expansion and sequencing using reverse transcriptase and a polymerase. See, Ausbel, Sambrook and Berger, *all supra*.

Oligonucleotides for *e.g.*, *in vitro* amplification methods, or for use as gene probes are typically chemically synthesized according to the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), *Tetrahedron Letts.*, 22(20):1859-1862, *e.g.*, using an automated synthesizer, as described in Needham-VanDevanter *et al.* (1984) *Nucleic Acids Res.*, 12:6159-6168. Purification of oligonucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson and Regnier (1983) *J. Chrom.* 255:137-149. The sequence of the synthetic oligonucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, *Methods in Enzymology* 65:499-560.

Genomic or cDNA libraries for the isolation of nucleic acids encoding Pfs25 or Pfs28 proteins, which are optionally cloned into the fusion proteins of the invention, are prepared according to standard techniques as described, for instance, in Sambrook, *supra*. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. Two kinds of vectors are commonly used for this purpose, bacteriophage lambda vectors and plasmids.

To prepare cDNA, mRNA from the parasite of interest is first isolated. Eukaryotic mRNA has at its 3' end a string of adenine nucleotide residues known as the poly-A tail. Short chains of oligo d-T nucleotides are then hybridized with the poly-A tails and serve as a primer for the enzyme, reverse transcriptase. This enzyme uses RNA as a template to synthesize a complementary DNA (cDNA) strand. A second DNA strand is then synthesized using the first cDNA strand as a template. Linkers are added to the double-stranded cDNA for insertion into a plasmid or phage vector for propagation in *E. coli*.

Identification of clones in either genomic or cDNA libraries harboring the desired nucleic acid segments is performed by either nucleic acid hybridization or



immunological detection of the encoded protein, if an expression vector is used. The bacterial colonies are then replica plated on a solid support, such as a nitrocellulose or nylon filter. The cells are lysed and probed with either oligonucleotide probes described above or with antibodies to the desired protein.

Other methods well known to those skilled in the art can also be used to identify desired genes. For example, the presence of restriction fragment length polymorphisms (RFLP) between wild type and mutant strains lacking a Pfs25 or Pfs28 polypeptide can be used. Amplification techniques, such as the polymerase chain reaction (PCR) can be used to amplify the desired nucleotide sequence. Sequences amplified by PCR can be purified from agarose gels and cloned into an appropriate vector according to standard techniques.

#### Expression and Purification of Fusion Proteins

Standard transfection methods are used to produce prokaryotic, mammalian, yeast or, insect, cell lines which express large quantities of the fusion polypeptide, which is then purified using standard techniques. The Pfs28 fusion proteins of the invention are purified from cells expressing the fusion proteins. Methods for purifying desired proteins are well known in the art and are not presented in detail here. For a review of standard techniques see, *Methods in Enzymology*, "Guide to Protein Purification", M. Deutscher, ed. Vol. 182 (1990), which is incorporated herein by reference. For instance, polypeptides can be purified using affinity chromatography, SDS-PAGE, and the like. See, also., Colley *et al.*, *J. Biol. Chem.* 264:17619-17622, 1989.

The nucleotide sequences encoding the fusion proteins of the invention are used to transfect host cells, which are modified according to standard techniques to yield fusion proteins. The polypeptides of the present invention can be readily designed and manufactured utilizing various recombinant DNA techniques well known to those skilled in the art. For example, the polypeptides can vary from the naturally-occurring sequence at the primary structure level by amino acid/insertions, substitutions, deletions, and the like. These modifications can be used in a number of combinations to produce the final modified protein chain.

The amino acid sequence variants can be prepared with various objectives in

mind, including facilitating purification and preparation of the recombinant polypeptide. Modified polypeptides are also useful for modifying plasma half life, improving therapeutic efficacy, and lessening the severity or occurrence of side effects during therapeutic use. The amino acid sequence variants are usually predetermined variants not found in nature but which exhibit similar immunogenic activity to naturally occurring Pfs28 or the antigen.

In general, modifications of the sequences encoding Pfs28 fusion proteins is readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis, PCR amplification using degenerate oligonucleotides, exposure of cells containing the nucleic acid to mutagenic agents or radiation, chemical synthesis of a desired oligonucleotide (*e.g.*, in conjunction with ligation and/or cloning to generate large nucleic acids) and other well-known techniques. *See*, Gilman and Smith (1979) *Gene* 8:81-97; Roberts *et al.* (1987) *Nature* 328:731-734 and Sambrook, Innis, Ausbel, Berger, Needham VanDevanter and Mullis, *supra*.

The particular procedure used to introduce the nucleic acids into a host cell for expression of the fusion protein is not critical. Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, spheroplasts, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (*see*, Berger, Ausbel and Sambrook, *all supra*).

A variety of commercially or commonly available vectors and vector nucleic acids can be converted into a vector of the invention by cloning a nucleic acid encoding a fusion protein of the invention into the commercially or commonly available vector. A variety of common vectors suitable for this purpose are well known in the art. For cloning in bacteria, common vectors include pBR322 derived vectors such as pBLUESCRIPT™, and  $\lambda$ -phage derived vectors. In yeast, vectors include Yeast Integrating plasmids (*e.g.*, YIp5) and Yeast Replicating plasmids (the YRp series plasmids) and pGPD-2. Expression in mammalian cells can be achieved using a variety of commonly available plasmids, including pSV2, pBC12BI, and p91023, as well as lytic virus vectors (*e.g.*, vaccinia virus, adeno virus, and baculovirus), episomal virus vectors (*e.g.*, bovine papillomavirus), and retroviral vectors (*e.g.*, murine retroviruses).

A nucleic acid subsequence encoding a selected fusion polypeptide is placed under the control of a promoter. An extremely wide variety of promoters are well known, and can be used in the vectors of the invention, depending on the particular application. Ordinarily, the promoter selected depends upon the cell in which the promoter is to be active. Other expression control sequences such as ribosome binding sites, transcription termination sites and the like are also optionally included. For *E. coli*, example control sequences include the T7, trp, or lambda promoters, a ribosome binding site and preferably a transcription termination signal. For eukaryotic cells, the control sequences typically include a promoter which optionally includes an enhancer derived from immunoglobulin genes, SV40, cytomegalovirus, *etc.*, and a polyadenylation sequence, and may include splice donor and acceptor sequences. In yeast, convenient promoters include GAL1,10 (Johnson and Davies (1984) *Mol. Cell. Biol.* 4:1440-1448) ADH2 (Russell *et al.* (1983) *J. Biol. Chem.* 258:2674-2682), PH05 (*EMBO J.* (1982) 6:675-680), and MF $\alpha$ l (Herskowitz and Oshima (1982) in *The Molecular Biology of the Yeast Saccharomyces* (eds. Strathern, Jones, and Broach) Cold Spring Harbor Lab., Cold Spring Harbor, N.Y., pp. 181-209). A multicopy plasmid with selective markers such as Leu-2, URA-3, Trp-1, and His-3 is also commonly used. A number of yeast expression plasmids such as YEp6, YEp13, YEp4 can be used as expression vectors. A gene of interest can be fused, *e.g.*, to any of the promoters in known yeast vectors. The above-mentioned plasmids have been fully described in the literature (Botstein *et al.* (1979) *Gene* 8:17-24; Broach, *et al.* (1979) *Gene*, 8:121-133). For a discussion of yeast expression plasmids, *see, e.g.*, Parents, B., *YEAST* (1985), and Ausbel, Sambrook and Berger, *all supra*).

Efficient expression and secretion in yeast is conveniently obtained using expression vectors based on those disclosed in Barr *et al.*, *J. Biol. Chem.* 263: 16471-16478, 1988, or U.S. Patent No. 4,546,082, which are incorporated herein by reference. In these vectors the desired sequences are linked to sequences encoding the yeast  $\alpha$ -factor pheromone secretory signal/leader sequence. Suitable promoters to use include the ADH2/GAPDH hybrid promoter as described in Cousens *et al.*, *Gene* 61:265-275 (1987), which is incorporated herein by reference. Yeast cell lines suitable for the present invention include BJ 2168 (Berkeley Yeast Stock Center) as well as other commonly available lines.

Any of a number of other well known cells and cell lines can be used to express the polypeptides of the invention. For instance, prokaryotic cells such as *E. coli* can be used. Eukaryotic cells include Chinese hamster ovary (CHO) cells, COS cells, mouse L cells, mouse A9 cells, baby hamster kidney cells, C127 cells, PC8 cells, and insect cells. The cells are optionally primary or immortalized cells isolated from a mammal or cultured from a patient. See, e.g., Freshney *et al.*, (Culture of Animal Cells, a Manual of Basic Technique, third edition Wiley-Liss, New York (1994)) and the references cited therein for a discussion of how to isolate and culture cells from patients. Alternatively, the cells can be those stored in a cell bank (e.g., a blood bank).

Following the growth of the recombinant cells and expression of the polypeptide, the culture medium is harvested for purification of the secreted protein. The media are typically clarified by centrifugation or filtration to remove cells and cell debris and the proteins are concentrated by adsorption to any suitable resin such as, for example, CDP-Sepharose, Asialoprothrombin-Sepharose 4B, or Q Sepharose, or by use of ammonium sulfate fractionation, polyethylene glycol precipitation, or by ultrafiltration. Other routine means known in the art are equally suitable. Further purification of the fusion polypeptide can be accomplished by standard techniques, for example, affinity chromatography, ion exchange chromatography, sizing chromatography or other protein purification techniques to obtain homogeneity. The purified fusion proteins are then used to produce immunological and/or pharmaceutical compositions.

#### Transmission-blocking Antibodies

A further aspect of the invention includes antibodies against the fusion Pfs25-Pfs28 polypeptides of the invention. The antibodies are useful for blocking transmission of malarial parasites. A demonstration of this transmission blocking activity is achieved by testing the transmission blocking activity in a standard membrane feeding assay. See, Quakyi (1987) *J. Immunol.* 139:4213-4221. The transmission blocking antibodies are also useful as reagents for the detection of proteins corresponding to the fusion protein (e.g., Pfs25, Pfs28, and Pfs25-Pfs28 fusion proteins) in biological samples. The antibodies of the invention are optionally polyclonal and are capable of blocking parasite transmission.

Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen (antigen), preferably a purified fusion polypeptide, a fusion polypeptide coupled to an appropriate carrier (*e.g.*, GST, keyhole limpet hemanocyanin, *etc.*), or a fusion polypeptide incorporated into an immunization vector, such as a recombinant vaccinia virus (*see*, U.S. Patent No. 4,722,848) is mixed with an adjuvant such as alum, and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired (*see, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY).

Antibodies, including binding fragments and single chain recombinant versions thereof, against whole or predetermined fusion protein subsequences are raised by immunizing animals, *e.g.*, with conjugates of the fragments with carrier proteins or adjuvants. The peptides are optionally coupled to a carrier protein (*e.g.*, as a part of the fusion protein), or are recombinantly expressed in an immunization vector. Antigenic determinants on selected peptides to which antibodies bind are typically 3 to 10 amino acids in length.

Monoclonal antibodies are optionally prepared from cells secreting the desired antibody. These antibodies are screened for binding to normal or modified polypeptides, or screened for agonistic or antagonistic activity, *e.g.*, activity mediated through a selected polypeptide. Specific monoclonal and polyclonal antibodies will usually bind a particular protein with a  $K_D$  of at least about .1 mM, more usually at least about 50  $\mu$ M, and preferably at least about 1  $\mu$ M or better. Such specific antibodies do not bind to structurally unrelated polypeptides.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, *etc.*, for use, *e.g.*, as an immunological reagent for the detection of Pfs25, Pfs28, or a fusion protein, or for affinity purification of such a protein. Description of techniques for preparing monoclonal

antibodies are found in, *e.g.*, Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane, *Supra*; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497. Summarized briefly, this method proceeds by injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secretes a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, retroviruses, or other methods known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells is enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate (preferably mammalian) host. The polypeptides and antibodies of the present invention are used with or without modification, and include chimeric antibodies such as humanized murine antibodies.

Other suitable techniques for antibody preparation involve selection of libraries of recombinant antibodies in phage or similar vectors (*see, e.g.*, Huse *et al.* (1989) *Science* 246: 1275-1281; and Ward, *et al.* (1989) *Nature* 341: 544-546; and Vaughan *et al.* (1996) *Nature Biotechnology*, 14: 309-314).

The antibodies of this invention are optionally used for affinity chromatography in isolating natural or recombinant Pfs 25 or Pfs 28 polypeptides. Columns are prepared, *e.g.*, with the antibodies linked to a solid support, *e.g.*, particles, such as agarose, Sephadex, or the like, where a cell lysate is passed through the column, washed, and treated with increasing concentrations of a mild denaturant, whereby purified polypeptides are released.

In one embodiment, the antibodies are used to screen expression libraries for

particular expression products such as homologous proteins to Pfs25 or Pfs28, *e.g.*, in an expression library from human, parasite, mammalian or insect tissue. Optionally, the antibodies in such a procedure are labeled with a moiety facilitating detection of antibody binding.

Transmission blocking antibodies are optionally humanized. Humanized (chimeric) antibodies are immunoglobulin molecules comprising a human and non-human portion. The antigen combining region (or variable region) of a humanized chimeric antibody is derived from a non-human source (*e.g.*, murine) and the constant region of the chimeric antibody (which confers biological effector function, such as cytotoxicity, to the immunoglobulin) is derived from a human source. The humanized chimeric antibody has the antigen binding specificity of the non-human antibody molecule and the effector function conferred by the human antibody molecule. A large number of methods of generating chimeric antibodies are well known to those of skill in the art (*see, e.g.*, U.S. Patent Nos: 5,502,167, 5,500,362, 5,491,088, 5,482,856, 5,472,693, 5,354,847, 5,292,867, 5,231,026, 5,204,244, 5,202,238, 5,169,939, 5,081,235, 5,075,431, and 4,975,369).

In another embodiment, this invention provides for fully human antibodies. Human antibodies consist entirely of characteristically human immunoglobulin sequences. The human antibodies of this invention can be produced in using a wide variety of methods (*see, e.g.*, Larrick *et al.*, U.S. Pat. No. 5,001,065, for a review). A general approach for producing human antibodies by trioma technology is described by Ostberg *et al.* (1983), *Hybridoma* 2: 361-367, Ostberg, U.S. Pat. No. 4,634,664, and Engelman *et al.*, U.S. Pat. No. 4,634,666. Other approaches include immunization of mice transformed to express human immunoglobulin genes, and phage display screening (Vaughan *et al. supra.*). Human antibodies are also made by immunizing a human with a fusion protein of the invention.

#### Immunogenic Compositions Comprising Pharmaceutically Acceptable Carriers

The nucleic acids, antibodies and polypeptides of the present invention are also useful as prophylactics, as immunogenic compositions, or as vaccine, for blocking transmission of malaria or other diseases caused by parasites. Compositions containing the nucleic acids, antibodies, polypeptides, or a mixture thereof are typically administered to a

mammal, giving rise to an immune response in the mammal, including the production of antibodies directed to the desired epitope. In the case of Pfs28-Pfs25 fusions, the antibodies block replication of the malarial parasite in an arthropod vector (typically a mosquito) when the antibodies are taken up by the vector with a blood meal. Preventing replication of the malarial parasite in the arthropod vector prevents transmission of the parasite by preventing the parasite from completing its life cycle. An amount of an immunogenic composition sufficient to elicit antibodies which inhibit transmission is defined to be an "immunologically effective dose."

Note that the immunogenic composition can be either a nucleic acid encoding a Pfs28 fusion protein (*e.g.*, a DNA vaccine), or a Pfs28 fusion protein. In addition, a prophylactic effect can be achieved by administering antisera raised to a Pfs28 fusion protein. In any case, the immunogenic or prophylactic composition typically further comprises one or more pharmaceutically acceptable excipient.

Isolated nucleic acid sequences coding for Pfs28 fusion proteins, or homologous polypeptides can also be used to transform viruses which transfect host cells in the susceptible organism. Live attenuated viruses, such as vaccinia or adenovirus, are convenient alternatives for the delivery of immunogenic compositions, because they are inexpensive to produce and are easily transported and administered. Vaccinia vectors and methods useful in immunization protocols are described in, *e.g.*, U.S. Patent No. 4,722,848, incorporated herein by reference.

Suitable viruses for use in the present invention include, but are not limited to, pox viruses, such as canarypox, cowpox viruses and vaccinia viruses, alpha viruses, adenoviruses and other animal viruses.

In the case of vaccinia virus (for example, strain WR), the sequence encoding the fusion polypeptides can be inserted into the viral genome by a number of methods including homologous recombination using a transfer vector, such as pTKgpt-OFIS as described in Kaslow *et al.*, (1991) *Science* 252:1310-1313.

Pfs28 fusion polypeptides, nucleic acids encoding the fusion polypeptides, or recombinant viruses of the present invention can be used in pharmaceutical and vaccine compositions that are useful for administration to mammals, particularly humans, to block transmission of a variety of infectious diseases. The compositions are suitable for single



administrations or a series of administrations. When given as a series, inoculations subsequent to the initial administration are given to boost the immune response and are typically referred to as booster inoculations.

The pharmaceutical compositions of the invention are intended for parenteral, topical, oral or local administration. Preferably, the pharmaceutical compositions are administered parenterally, *e.g.*, intravenously, subcutaneously, intradermally, or intramuscularly. Most commonly, administration is by subcutaneous or intramuscular administration. Thus, the invention provides compositions for parenteral administration that comprise a solution of the polypeptides, nucleic acids or antibodies described above, dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. These compositions are sterilized by conventional, well known sterilization techniques, and can be sterile filtered. The resulting aqueous solutions are packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions contain pharmaceutically acceptable auxiliary substances to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid carriers are optionally used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient (fusion protein, nucleic acid, viral vector, or antibody), and more preferably at a concentration of 25%-75%.

For aerosol administration, the polypeptides or recombinant viruses are preferably supplied in finely divided form along with a surfactant and propellant. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant.

Representative of such agents are the esters or partial esters of fatty acids containing from 6

to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. A carrier can also be included, as desired, as with, *e.g.*, lecithin for intranasal delivery.

In therapeutic applications, polypeptides, nucleic acids, or recombinant viruses of the invention are administered to a patient in an amount sufficient to prevent parasite development in the arthropod and thus block transmission of the disease. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend on, *e.g.*, the particular polypeptide or virus, the manner of administration, the weight and general state of health of the patient, and the judgment of the prescribing physician. Subcutaneous or intramuscular doses for fusion proteins will typically range  $1\mu\text{g}$  to  $200\mu\text{g}$  for a 70kg patient in generally good health. Subcutaneous or intramuscular doses for naked nucleic acid (typically DNA encoding a fusion protein) will range from  $.1\mu\text{g}$  to  $500\mu\text{g}$  for a 70kg patient in generally good health. Subcutaneous or intramuscular doses for viral vectors comprising the fusion proteins of the invention will range from  $1 \times 10^5$  pfu to  $1 \times 10^9$  for a 70kg patient in generally good health.

The vaccines of the invention contain as an active ingredient an immunogenically effective amount of the polypeptides, nucleic acids, or recombinant viruses as described herein. Useful carriers are well known in the art, and include, *e.g.*, thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(D-lysine:D-glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are also advantageously used to boost an immune response.

#### Discussion of the Sequences In the Examples

The information for the nucleic acid sequences in the examples below are presented as DNA sequence information. One of skill will readily understand that the sequences also describe RNAs encoded by the sequence (*e.g.*, by substitution of T residues

including silent substitutions of the sequences. While only a single strand of sequence information is shown, one of skill will immediately appreciate that the complete corresponding complementary sequence is fully described by comparison to the given sequences.

A variety of conservatively modified variations of the amino acid sequences provided in the examples are also described herein. One of skill will also recognize that a variety of nucleic acid sequences encode each of the polypeptides due to the codon degeneracy present in the genetic code. Each of the nucleic acids which encodes the given polypeptide is described by comparison to the amino acid sequence and translation via the genetic code. In preferred embodiments, a nucleic acid which encodes a particular polypeptide is optimized for expression in a particular cell type, such as yeast, by reference to sequence codon bias tables and substitution of a given sequence with a sequence which encodes the same polypeptide using codons preferred for the cell type.

### EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially similar results.

#### Example 1: TBV25-Pfs28 Constructs

Constructs contain the C-terminus of the prepro- $\alpha$ -factor signal sequence, TBV25 (Pfs25 gene in yeast preferred codons from Ala<sub>22</sub> to Thr<sub>193</sub>), a flexi-linker (Gly<sub>3</sub>-Pro-Gly<sub>3</sub>), Pfs28 or TBV28 (Pfs28 gene in yeast preferred codons, a linker (GlyPro) and His<sub>6</sub>).

**TBV25-Pfs28B** Sequences encoding TBV25 from Ala<sub>22</sub> to Thr<sub>193</sub> was amplified by PCR using oligonucleotides 5'TBV25 5'-

***GGGGTACCTTTGGATAAAAGAGCTAAGGTC***ACTGTCGAC-3' (introduced  $\alpha$ -factor leader sequence and restriction site are shown in italics and bold respectively) and 3'TBV25 5'-***CCCCCGGGACCACCGGTACAAATGGAAGATTCTTG***-3' (introduced flexi-linker and restriction site are shown in italics and bold respectively) and digested with *KpnI* and *SmaI*. Sequences encoding Pfs28 from Val<sub>24</sub> to Pro<sub>179</sub> was amplified by PCR using

oligonucleotides 5'Pfs28

5'-GGT**CCCCGGGGGTGGT**GTTACTGAAAATACAATATGT-3' (introduced flexi-linker and restriction site are shown in *italics* and **bold** respectively) and

5'-AAACTAGTGGTGGTGGTGGTGGT**GAGGATCCTCTTTACATGTATAATA**-3' (introduced His<sub>6</sub> is *italicized* and new restriction site appear in **bold**) and digested with *Sma*I and *Spe*I. The pIXY shuttle plasmid was digested with *Kpn*I and *Spe*I. A three-way ligation was performed using the digested PCR products and vector.

#### Tbv25-Pfs28B

```

GCTAAGGTCACTGTCGACACCGTCTGTAAGAGAGGTTTCTTGATTCAAATGTCC 54
A K V T V D T V C K R G F L I Q M S

GGTCACTTGGAATGTAAGTGTGAAAACGACTTGGTCTTGGTTAACGAAGAACT 108
G H L E C K C E N D L V L V N E E T

TGTGAAGAAAAGGTCTTGAAGTGTGACGAAAAGACTGTCAACAAGCCATGTGGT 162
C E E K V L K C D E K T V N K P C G

GACTTCTCTAAGTGTATCAAGATCGATGGTAACCCAGTCTCTTACGCCTGTAAG 216
D F S K C I K I D G N P V S Y A C K

TGTAACCTGGGGTTACGATATGGTCAACAACGTCTGTATTCCAAACGAATGTAAG 270
C N L G Y D M V N N V C I P N E C K

CAAGTTACCTGTGGTAACGGTAAGTGTATCTTGGATACTTCCAACCCAGTTAAG 324
Q V T C G N G K C I L D T S N P V K

ACCGGTGTTTGTCTTGTAAACATTGGTAAGGTCCCAAACGTTCAAGACCAAAAC 378
T G V C S C N I G K V P N V Q D Q N

AAGTGTCTTAGAGACGGTGAACTAAGTGTTCCTTGAAGTGTTTGAAGGAACAA 432
K C S R D G E T K C S L K C L K E Q

GAAACCTGTAAGGCTGTTGACGGTATTTACAAGTGTGACTGTAAGGATGGTTTC 486
E T C K A V D G I Y K C D C K D G F

ATCATTGACCAAGAATCTTCCATTTGTACCGGTGGTGGTCCCCGGGGGAGGTGTT 540
I I D Q E S S I C T G G G P G G G V

ACTGAAAATACAATATGTAAATATGGTTATTTAATTCAGATGAGTAATCATTAT 594
T E N T I C K Y G Y L I Q M S N H Y

GAATGTAAGTGTATTGAAGGATATGTATTAATAAATGAGGACACGTGTGGAAAA 648
E C K C I E G Y V L I N E D T C G K

AAAGTAGTCTGTGATAAAGTTGAAAATTCATTTAAAGCTTGTGATGAATACGCT 702
K V V C D K V E N S F K A C D E Y A

TACTGTTTCGATTAGGAAATAAGAATAATGAAAAACAGATAAAATGTATGTGC 756
Y C F D L G N K N N E K Q I K C M C

AGAACAGAATATACTTTAACTGCTGGAGTATGTGTTCTTAATGTTTGTGCGAGAT 810
R T E Y T L T A G V C V P N V C R D

AAAGTATGTGGTAAAGGAAAATGTATAGTAGATCCTGCAAATCTTTAACACAT 864
K V C G K G K C I V D P A N S L T H

ACATGCTCATGCAATATAGGTACCATACTTAACCAGAATAAATTATGTGATATA 918

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T C S C N I G T I L N Q N K L C D I  
 CAAGGTGATACACCATGTTTCATTAAATGTGCAGAAAATGAAGTGTGTACATTA 972  
 Q G D T P C S L K C A E N E V C T L  
 GAAGGAAATTATTATACATGTAAAGAGGATCCT**CACCACCACCACCACCAC** 1023  
 E G N Y Y T C K E D P H H H H H H

The flexi-linker (Gly<sub>3</sub>ProGly<sub>3</sub>) in between TBV25 and TBV28 and His<sub>6</sub> at the C-terminus appear in bold.

**TBV25-Pfs28C** Sequences encoding TBV25 from Ala<sub>22</sub> to Thr<sub>193</sub> was amplified by PCR using oligonucleotides 5'TBV25

5'-**GGGGTACCTTTGGATAAAAGAGCTAAGGTC**ACTGTCGAC-3' (introduced  $\alpha$ -factor leader sequence and restriction site are shown in italics and bold respectively) and 3'TBV25 5'-**CCCCCGGGACCACCACCGGTACAAATGGAAGATTCTTG**-3' (introduced flexi-linker and restriction site are shown in italics and bold respectively) and digested with *KpnI* and *SmaI*. Sequences encoding Pfs28 from Val<sub>24</sub> to Ser<sub>196</sub> was amplified by the PCR using oligonucleotides 5'Pfs28

5'-GGT**CCCCGGGGGTGGTGT**TA**CTGAAAATACAATATGT**-3' (introduced flexi-linker and restriction site are shown in italics and bold respectively) and

5'-AACTAGTGGTGGTGGTGGTGGTGGGGCC**ACTATATGATGTATCAGCCTG**-3' (introduced linker and His<sub>6</sub> are italicized and new restriction site appear in bold) and digested with *SmaI* and *SpeI*. The pIXY shuttle plasmid was digested with *KpnI* and *SpeI*.

A three-way ligation was performed using the digested PCR products and vector.

**TBV25-Pfs28C**

GCTAAGGTC**ACTGTCGACACCGTCTGTAAGAGAGGTTCTTGATTCAAATGTCC** 54  
 A K V T V D T V C K R G F L I Q M S

GGTCACTTGGAAATGTAAGTGTGAAAACGACTTGGTCTTGGTTAACGAAGAACT 108  
 G H L E C K C E N D L V L V N E E T

TGTGAAGAAAAGGTCTTGAAGTGTGACGAAAAGACTGTCAACAAGCCATGTGGT 162  
 C E E K V L K C D E K T V N K P C G

GACTTCTCTAAGTGTATCAAGATCGATGGTAACCCAGTCTCTTACGCCTGTAAG 216  
D F S K C I K I D G N P V S Y A C K

TGTAACCTGGGTTACGATATGGTCAACAACGTCTGTATTCCAAACGAATGTAAG 270  
C N L G Y D M V N N V C I P N E C K

CAAGTTACCTGTGGTAACGGTAAGTGTATCTTGGATACTTCCAACCCAGTTAAG 324  
Q V T C G N G K C I L D T S N P V K

ACCGGTGTTTGTCTTGTAAACATTGGTAAGGTCCCAAACGTTCAAGACCAAAAC 378  
T G V C S C N I G K V P N V Q D Q N

AAGTGTCTAGAGACGGTGAACTAAGTGTTCCTTGAAGTGTITGAAGGAACAA 432  
K C S R D G E T K C S L K C L K E Q

GAAACCTGTAAGGCTGTTGACGGTATTTACAAGTGTGACTGTAAGGATGGTTTC 486  
E T C K A V D G I Y K C D C K D G F

ATCATTGACCAAGAATCTTCCATTGTACCGGTGGTGGTCCCGGGGAAGTGT 540  
I I D Q E S S I C T G G G P G G G V

ACTGAAAATACAATATGTAATATGGTTATTTAATTCAGATGAGTAATCATTAT 594  
T E N T I C K Y G Y L I Q M S N H Y

GAATGTAAGTGTATTGAAGGATATGTATTAATAAATGAGGACACGTGTGGAAAA 648  
E C K C I E G Y V L I N E D T C G K

AAAGTAGTCTGTGATAAAGTTGAAAATTCATTTAAAGCTTGTGATGAATACGCT 702  
K V V C D K V E N S F K A C D E Y A

TACTGTTTCGATTTAGGAAATAAGAATAATGAAAAACAGATAAAATGTATGTGC 756  
Y C F D L G N K N N E K Q I K C M C

AGAACAGAATATACTTTAACTGCTGGAGTATGTGTTCTAATGTTTGTGCGAGAT 810  
R T E Y T L T A G V C V P N V C R D

AAAGTATGTGGTAAGGAAAATGTATAGTAGATCCTGCAAATTCCTTAACACAT 864  
K V C G K G K C I V D P A N S L T H

ACATGCTCATGCAATATAGGTACCATACTTAACCAGAATAAATTATGTGATATA 918  
T C S C N I G T I L N Q N K L C D I

CAAGGTGATACACCATGTTCAATAAATGTGCAGAAAATGAAGTGTGTACATTA 972  
Q G D T P C S L K C A E N E V C T I

GAAGGAAATTATTATACATGTAAAGAAGATCCTTCATCTAACGGAGGAGGAAAT 1026  
E G N Y Y T C K E D P S S N G G G N

ACTGTGGACCAAGGCTGATACATCATATAGTGGGCCCCACCACCACCACCACCAC 1080  
T V D Q A D T S Y S G P H H H H H H

The flexi-linker (Gly<sub>3</sub>ProGly<sub>3</sub>) in between TBV25 and TBV28, the linker (GlyPro) and His<sub>6</sub> at the C-terminus appear in bold.

**TBV25-Pfs28D** Sequences encoding TBV25 from Ala<sub>22</sub> to Thr<sub>193</sub> was amplified by PCR using oligonucleotides 5'TBV25

5'-GGGGTACCTTTGGATAAAAGAGCTAAGGTCACTGTCGAC-3' (introduced  $\alpha$ -factor leader sequence and restriction site are shown in italics and bold respectively) and 3'TBV25

5'-CCCCCGGGACCACCACCGGTACAAATGGAAGATTCTTG-3' (introduced flexi-linker and restriction site are shown in italics and bold respectively) and digested with *KpnI* and *SmaI*. Sequences encoding Pfs28 from Val<sub>24</sub> to Cys<sub>175</sub> was amplified by PCR using oligonucleotides 5'Pfs28

5'-GGTCCCGGGGGTGGTGTACTGAAAATACAATATGT-3' (introduced flexi-linker and restriction site are shown in italics and bold respectively) and

5'-AAACTAGTGGTGGTGGTGGTGGGGCCCATGTATAATAATTCCTTC-3' (introduced linker and His<sub>6</sub> are italicized and new restriction site appear in bold) and digested with *SmaI* and *SpeI*. The pIXY shuttle plasmid was digested with *KpnI* and *SpeI*. A three-way ligation was performed using the digested PCR products and vector. **TBV25-Pfs28D**

GCTAAGGTCACTGTCGACACCGTCTGTAAGAGAGGTTTCTTGATTCAAATGTCC 54  
A K V T V D T V C K R G F L I Q M S

GGTCACTTGGAATGTAAGTGTGAAAACGACTTGGTCTTGGTTAACGAAGAAACT 108  
G H L E C K C E N D L V L V N E E T

TGTGAAGAAAAGGTCTTGAAGTGTGACGAAAAGACTGTCAACAAGCCATGTGGT 162  
C E E K V L K C D E K T V N K P C G

GACTTCTCTAAGTGTATCAAGATCGATGGTAACCCAGTCTCTTACGCCTGTAAG 216  
D F S K C I K I D G N P V S Y A C K

TGTAACCTGGGTACGATATGGTCAACAACGTCTGTATTCCAAACGAATGTAAG 270  
C N L G Y D M V N N V C I P N E C K

CAAGTTACCTGTGGTAACGGTAAGTGTATCTTGGATACTTCCAACCCAGTTAAG 324  
Q V T C G N G K C I L D T S N P V K

ACCGGTGTTTGTCTTGTAAACATTGGTAAGGTCCCAAACGTTCAAGACCAAAAC 378  
T G V C S C N I G K V P N V Q D Q N

AAGTGTCTAGAGACGGTGAAACTAAGTGTTCCTTGAAGTGTGTAAGGAACAA 432  
K C S R D G E T K C S L K C L K E Q

GAAACCTGTAAGGCTGTTGACGGTATTTACAAGTGTGACTGTAAGGATGGTTTC 486  
E T C K A V D G I Y K C D C K D G F

ATCATTGACCAAGAATCTTCATTTGTACCGGTGGTGGTCCCGGGGAGGTGTT 540  
I I D Q E S S I C T G G G P G G G V

ACTGAAAATACAATATGAAATATGGTTATTTAATTCAGATGAGTAATCATTAT 594  
T E N T I C K Y G Y L I Q M S N H Y

GAATGTAAGTGATTGAAGGATATGTATTAATAAATGAGGACACGTGTGAAAA 648  
E C K C I E G Y V L I N E D T C G K

AAAGTAGTCTGTGATAAAGTTGAAAATTCATTTAAAGCTTGTGATGAATACGCT 702  
K V V C D K V E N S F K A C D E Y A

TACTGTTTCGATTTAGGAAATAAGAATAATGAAAAACAGATAAAATGTATGTGC 756  
Y C F D L G N K N N E K Q I K C M C

AGAACAGAATATACTTTAACTGCTGGAGTATGTGTTCTAATGTTTGTGAGAT 810  
R T E Y T L T A G V C V P N V C R D

AAAGTATGTGGTAAAGGAAAATGTATAGTAGATCCTGCAAATTCCTTAACACAT 864  
K V C G K G K C I V D P A N S L T H

ACATGCTCATGCAATATAGGTACCATACTTAACCAGAATAAATTATGTGATATA 918  
T C S C N I G T I L N Q N K L C D I

CAAGGTGATACACCATGTTCAATAAATGTGCAGAAAATGAAGTGTGTACATTA 972  
Q G D T P C S L K C A E N E V C T I

GAAGGAAATTATTATACATGTGGGCCCCACCACCACCACCACCACCAC 1017  
E G N Y Y T C G P H H H H H H

The flexi-linker (Gly<sub>3</sub>ProGly<sub>3</sub>) in between TBV25 and TBV28, the linker (GlyPro) and His<sub>6</sub> at the C-terminus appear in bold.

**TBV28 CONSTRUCT** The following oligonucleotides were used to construct synthetic TBV28 (Pfs28 in yeast-preferred codons from Val<sub>24</sub> to Ser<sub>196</sub>):

**OLIG01**

5'-

GGGGTACCTTTGGATAAAAAGAGTTACTGAAAACACTATTTGTAAGTACGG  
TTACTTGATTCAAATGTCTAACCACCTACGAATGTAAG - 3'

**OLIG02**

5'-TGTATTGAAGGTTACGTTTTGATTAAACGAGGACACTTGTGGTAAGAAG  
GTTGTTTGTGACAAGGTTGAAAACCTTTCAAGGCCTGT - 3'

**OLIG03**

5'-GATGAATACGCTTACTGTTTCGATTTGGGTAACAAGAACAACGAAAAG  
CAAATTAAGTGTATGTGTAGAACTGAATACACTTTGACT - 3'

**OLIG04**

5'-GCTGGTGTGTTGTGTTCCAAACGTTTGTAGAGACAAGGTTTGTG  
GTAAGGGTAAGTGTATTGTTGATCCAGCTAACTCTTTGACTCAC - 3'

**OLIG05**

5'-ACTTGTTCTTGTAACATTGGTACTATTTTGAACCAAAACAAGTTG  
TGTGATATCCAAGGTGATACTCCATGTTCTTTGAAGTGTGCT - 3'

**OLIG06**

5'-GAAAACGAAGTCTGTACTTTGGAAGGTAACACTACTACACTTGTAAGGAA



GACCCATCTTCTAACGGTGGTGGTAACACTGTCGACCAA - 3'

**OLIG07**

5'-GCCAAGCTTGGGCCCAGAGTAAGAAGTATCAGCTTGGTCGACAGTGTT  
ACCACCACCGTTAGAAGATGGGTCTTCCTTACAAGTGTA - 3'

**OLIG08**

5'-GTAGTTACCTTCCAAAGTACAGACTTCGTTTTTCAGCACACTTCAAA  
GAACATGGAGTATCACCTTGGATATCACACAACCTTGTTTTG - 3'

**OLIG09**

5'-GTTCAAAATAGTACCAATGTTACAAGAACAAGTGTGAGTCAAAG  
AGTTAGCTGGATCAACAATACACTTACCCTTACCACAAACCTT - 3'

**OLIGO10**

5'-GTCTCTACAAACGTTTGGAACACAAACACCAGCAGTCAAAGTGT  
ATTCAGTTCTACACATACTTAATTTGCTTTTCGTTGTTCTT - 3'

**OLIG011**

5'-GTTACCCAAATCGAAACAGTAAGCGTATTCATCACAGGCCTT  
GAAAGAGTTTTCAACCTTGTCAAAACAACCTTCTTACCACAAGT-3'

**OLIG012**

5'-GTCCTCGTTAATCAAAACGTAACCTTCAATACACTTACATTCGTAG  
TGGTTAGACATTTGAATCAAGTAACCGTACTTACAAATAGT-3'

**OLIG013**

5'-GCTGATACTTCTTACTCTGGGCCCCAAGCTTGGC-3'

**OLIG014**

5'-GTTTTAGTAAGTCTTTTATCCAAAGGTACCCC - 3'

**28OLIG01**

5'-CAAGGCCTGTGATGAATACGCTTACTGTTTCGATTTGGGTAACAAGAA  
CAACGAAAAGCAAATTAAGTGTATG-3'

**28OLIG02**

5'-CTTGGATATCACACAACCTTGTTTTGGTTCAAAATAGTACCAATGTTACA  
AGAACAAG-3'

**5STU**

5'-TTCAAGGCCTGTGATGAATACGCT - 3'

**3ECO**

5'-TTGGATATCACACAACCTTGTTTTG - 3'

**5TBV28**

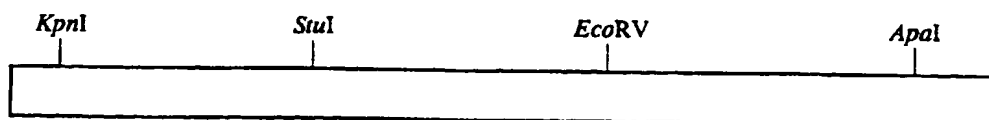
5'-GGTCCCGGGGGTGGTGTACTGAAAACACTATTTGT-3'

**3TBV28**

5'-AAACTAGTGGTGGTGGTGGTGGTGGGGCCCAGAGTAAGAAGTATCAGC  
TTG-3'

**3APA**

5'-CTTGGGCCCAGAGTAAGAAGTATCAGCTTGGTCGACAGTGTT-3'



Oligonucleotides 1, 2, 11, 8 (7.5 pmoles each) were phosphorylated,

annealed and ligated together. The annealed template was amplified by PCR using Oligo 1 and Oligo 11, filled-in, phosphorylated and ligated into *Sma*I BAP-treated pUC 18. The clone with the correct sequence was referred to as KS.

Oligonucleotides 2, 3, 4, 5, 8, 9, 10, 11 (7.5 pmoles each) were phosphorylated, annealed and ligated together. The annealed template was amplified by PCR using Oligo2 and Oligo8, filled-in, phosphorylated and ligated into *Sma*I BAP-treated pUC 18. Mistakes within the cloned sequence were corrected by PCR using shorter oligonucleotides 28Oligo 1 and 28Oligo 2. The PCR product was filled-in, phosphorylated and ligated into *Sma*I BAP-treated pUC 18. New mistakes within the cloned sequence was corrected by another PCR using shorter oligonucleotides 5Stu and 3Eco. The clone with the correct sequence was referred to as SE.

Oligonucleotides 5, 8, 6, 7, 13 (~7.5 pmoles each) were annealed, digested with *Eco*RV and *Apa*I while oligonucleotides 1-5, 8-12, 14 (~10 pmoles each) were annealed, digested with *Kpn*I and *Eco*RV separately. The products of the 2 digestions were ligated together and amplified by PCR using oligonucleotides 5TBV28 and Oligo 7. The PCR product was filled-in, phosphorylated and ligated into *Sma*I BAP-treated pUC 18. The clone with the correct sequence from the *Eco*RV site to the *Apa*I site was referred to as AE.

Sequential cloning proceeded as follows: SE clone was digested with *Stu*I *Pst*I-cut and the released insert was ligated into *Stu*I and dephosphorylated KS. The recombinant was then cut with *Eco*RV *Pst*I and dephosphorylated and ligated with *Eco*RV *Pst*I insert from the AE clone. The resultant recombinant contains full length TBV28 (Val<sub>24</sub> to Ser<sub>196</sub>).

#### **TBV25-28 Construct**

TBV28 was amplified by PCR using oligonucleotides 5'TBV28 5'-GGTCCCGGGGGTGGTGTACTGAAAACACTATTTGT-3' (introduced *Sma*I site appear in bold) and 3'TBV285'-AAACTAGTGGTGGTGGTGGTGGTGGGCCCCAGAGTAAGAAGTATCAGCTTG-3' (introduced linker sequence, GlyPro and His<sub>6</sub> are italicized and new restriction site appear in bold) digested with *Sma*I *Spe*I and ligated into *Sma*I *Spe*I-cut dephosphorylated pIXY925 recombinant plasmid which

contains the TBV25-Pfs28C construct. The new construct pIXY925' contained a deletion at the 3' end and was corrected by PCR using oligonucleotides 5Stu and 3Apa. The PCR product was filled-in, phosphorylated and ligated into *SmaI* BAP-treated pUC 18. A *StuI* *Apal* insert was released and gel-purified from the correct clone. A *KpnI* *Apal* insert was released and gel-purified from pIXY925'. A three-way ligation was performed using the two purified inserts and the *SmaI* *SpeI*-cut dephosphorylated pIXY925.

### TBV25-28: YEAST-PREFERRED CODONS

GAGGCCGAGGCTAAGGTCACCTGTCGACACCGTCTGTAAGAGAGGTTTCTTGATT 54

E A E A K V T V D T V C K R G F L I

CAAATGTCCGGTCACTTGGAATGTAAGTGTGAAAACGACTTGGTCTTGGTTAAC 108

Q M S G H L E C K C E N D L V I V N

GAAGAACTTGTGAAGAAAAGTCTTGAAGTGTGACGAAAAGACTGTCAACAAG 162

E E T C E E K V I K C D E K T V N K

CCATGTGGTGACTTCTCTAAGTGTATCAAGATCGATGGTAACCCAGTCTCTTAC 216

P C G D F S K C I K I D G N P V S Y

GCCTGTAAGTGTAACTTGGGTTACGATATGGTCAACAACGTCTGTATTCCAAAC 270

A C K C N L G Y D M V N N V C I P N

GAATGTAAGCAAGTTACCTGTGGTAACGGTAAGTGTATCTTGGATACTTCCAAC 324

E C K Q V T C G N G K C I L D T S N

CCAGTTAAGACCGGTGTTTGTCTTGTAAACATTGGTAAGGTCCCAAACGTTCAA 378

P V K T G V C S C N I G K V P N V Q

GACCAAAACAAGTGTCTAGAGACGGTGAAACTAAGTGTTCCTTGAAGTGTGTTG 432

D Q N K C S R D G E T K C S L K C L

AAGGAACAAGAAACCTGTAAGGCTGTTGACGGTATTTACAAGTGTGACTGTAAG 486

K E Q E T C K A V D G I Y K C D C K

GATGGTTTCATCATTGACCAAGAATCTTCCATTTGTACCGGTGGTGGTCCCCGGG 540

D G F I I D O E S S I C T G G G P G

GGTGGTGTACTGAAAACACTATTGTAACTACGGTTACTTGATTCAAATGTCT 594

G G V T E N T I C K Y G Y L I Q M S

AACCACTACGAATGTAAGTGATTGAAGGTTACGTTTGTGATTAACGAGGACACT 648

N H Y E C K C I E G Y V L I N E D T

TGTGGTAAGAAGGTTGTTTGTGACAAGGTTGAAAACCTCTTCAAGGCCTGTGAT 702

C G K K V V C D K V E N S F K A C D

GAATACGCTTACTGTTTCGATTGGGTAACAAGAACAACGAAAAGCAAATTAAG 756

E Y A Y C F D L G N K N N E K Q I K

TGTATGTGTAGAACTGAATACACTTTGACTGCTGGTGTGTTGTGTTCCAAACGTT 810

C M C R T E Y T L T A G V C V P N V

TGTAGAGACAAGGTTTGTGGTAAGGGTAAGTGATTGTTGATCCAGCTAACTCT 864

C R D K V C G K G K C I V D P A N S

TTGACTCACACTTGTCTTGTAACTTGGTACTATTTGAACCAAACAAGTTG 918

L T H T C S C N I G T I L N Q N K L

TGTGATATCCAAGGTGATACTCCATGTTCTTTGAAGTGTGCTGAAAACGAAGTC 972

C D I Q G D T P C S L K C A E N E V

TGTACTTTGGAAGGTAAGTACTTGTAAAGGAAGACCCATCTTCTAACGGT 1026

C T L E G N Y Y T C K E D P S S N G

GGTGGTAACACTGTCGACCAAGCTGATACTTCTTACTCTGGGCCCCACCACCAC 1080

G G N T V D Q A D T S Y S G P H H H

CACCACCAC

1089

H H H

The flexi-linker (Gly<sub>3</sub>ProGly<sub>3</sub>) in between TBV25 and TBV28, the linker (GlyPro) and

His<sub>6</sub> at the C-terminus appear in bold italics. The TBV25 sequence (Ala<sub>22</sub> to Thr<sub>193</sub>) is underlined. Yeast-preferred codons in TBV28 are shown in bold.

**Example 2: Preparation of Master Cell Bank for Tbv25-28-vk1**

**Strain History:** The recombinant plasmid, pIXY925 TBV25-TBV28, was electroporated into the VK1 strain of *Saccharomyces cerevisiae* and a single colony selected for further analysis as follows.

**Plasmid preparation:**

pIXY925 is a derivative of pIXY154 and consists of a modified ADH2 promoter (a tract of 54 adenines added), the Glu-Ala-Glu-Ala insertion after the KEX-2 cleavage site in the  $\alpha$ -factor pre-pro secretory sequence (contains a *Kpn* I restriction site), and encodes TBV25 (yeast codon preference from Ala<sub>22</sub> to Thr<sub>193</sub>), a linker sequence (Gly<sub>3</sub>-Pro-Gly<sub>3</sub>), Pfs28 (malaria codon preference from Val<sub>24</sub> to Ser<sub>196</sub>), and Gly-Pro-His<sub>6</sub> (contains an *Apa* I restriction site).

The parent vector portion of pIXY925 was digested with *Kpn* I and *Apa* I, dephosphorylated with calf intestine alkaline phosphatase and purified from a TAE-agarose gel using GeneClean™.

A PCR-amplified DNA fragment, encoding Pfs28 Ala<sub>73</sub> to Ser<sub>196</sub> in yeast preferred codons, was previously blunt-end cloned in *Sma* I-digested pUC18 (referred to as Miniprep clone 2 (MP2)). The fragment was sequenced to confirm that no undesired mutations had occurred. The 376 bp DNA fragment encoding Pfs28 Ala<sub>73</sub> to Ser<sub>196</sub> in yeast preferred codons was recovered from MP2 by digestion with *Apa* I and *Stu* I, TAE-agarose gel electrophoresis and GeneClean™.

A 708 bp *Kpn* I and *Stu* I DNA fragment, encoding the Kex2 cleavage site with a Glu-Ala-Glu-Ala insert, TBV25 (yeast codon preference from Ala<sub>22</sub> to Thr<sub>193</sub>), a linker sequence (Gly<sub>3</sub>-Pro-Gly<sub>3</sub>), TBV28 (yeast codon preference from Val<sub>24</sub> to Ala<sub>73</sub>), was recovered from plasmid pEAEA TBV25-28, and TAE-agarose gel purified using GeneClean™.

A three-way ligation of the purified DNA fragments from above was performed and 1  $\mu$ L of the ligation mixture electroporated into DH10B *E. coli*.

Plasmid DNA recovered by miniprep of the resulting clones from step 4 were analyzed by restriction enzyme digestion with *Stu I* and *Eco RV*. Recombinant plasmids were identified by the release of a 230 bp DNA fragment. Clone 11 was identified as having the predicted restriction enzyme fragments.

**Host cell preparation, electroporation, clone selection and storage:**

VK1 cells from VK1 Production Seed Lot

950912BPR/PSL/YST/VK1/001 were electroporated with plasmid from Clone 11.

A patch test for expression of TBV25H using mAb4B7 was performed to select recombinant VK1 clone VK1 Clone 3a pIXY925 TBV25-TBV28.

Glycerol stocks of the selected clone was prepared and stored at -70°C. One of the stocks was used in preparation of a master cell bank.

**Example 3: Summary of Transmission Blocking Activity**

**Fermentation**

Fermentation procedure was essentially as described by Kaslow & Shiloach (1994) *Biotechnology* 12:494-499. A 1 ml frozen seed lot was thawed and used to inoculate 500 ml of expansion medium (8% glucose, 1% yeast nitrogen base, 2% acid-hydrolyzed casamino acids, 400 mg/L adenine sulfate, 400 mg/L uracil) in a Tunair baffled shaker flask. The cells were grown overnight at 30°C with shaking at 250 rpm for 20-40 hr. The overnight growth in expansion medium was used to inoculate 3-3.5 L of fermentation media (0.5% glucose, 1% yeast extract, 1% yeast nitrogen base, 2% acid-hydrolyzed casamino acids, 400 mg/L adenine sulfate, 400 mg/L uracil). The Bioflo-III fermentor was set to keep pH at 5.02, temperature at 25°C and dissolved oxygen at or above 60% by agitation between 360 and 1000 rpm. A glucose-rich nutrient medium (25% glucose, 1% yeast extract, 1% yeast nitrogen base, 2% acid-hydrolyzed casamino acids, 0.5 g/L adenine sulfate, 0.5 g/L uracil, 2.5 g/L MgSO<sub>4</sub>) was fed continuously at a rate of 25 ml/hr for approximately 40 hr. 25% NH<sub>4</sub>OH was fed to keep pH at 5.02. When OD<sub>600</sub> of the culture reached 50 units, the carbon source was switched from glucose to 30% ethanol, 20% glycerol to induce protein secretion for 10-16 hr.

**Protein Purification**

The culture supernatant was recovered by centrifugation and filter-sterilized through a 0.45  $\mu$ m cellulose acetate membrane (Nalgene). The sterile medium was concentrated to 350 mLs using an Amicon tangential ultrafiltration apparatus fitted with a YD 10 spiral hollow fiber filter (Amicon), and then continuously dialyzed with 1.5 L 2X PBS pH 7.4. The retentate was incubated with Ni-NTA agarose with shaking at 4°C overnight. After overnight incubation, the suspension was transferred to a column and the resin was washed sequentially with 2X PBS pH 7.4, 2X PBS pH 6.8 and 1X PBS pH 6.4. The protein was eluted from the resin using 0.250 M NaAcetate pH 4.5 and analyzed by SDS-PAGE. Further purification was performed by size-exclusion chromatography using a Pharmacia Superdex-75 column to which 1X PBS pH 7.4 was applied at a flow rate of 1mL/min. One mL fractions were collected and analyzed by SDS-PAGE. Fractions containing the ~39kD fusion protein were pooled and protein concentration was determined by BCA (Pierce) using bovine serum albumin as the standard.

#### **Immunization and Transmission-blocking Assay**

Purified fusion proteins were adsorbed to alum (Superfos Biosector a/s) for 30 min at room temperature with continuous rocking. The suspensions were then stored at 4°C until used to vaccinate mice by the intraperitoneal route. The mice received three injections at three week intervals. Transmission-blocking activity was assayed as described previously Quakyi *et al* (1987) J. Immunol. 139: 4213-4217. Briefly, test sera were mixed with mature *in vitro*-cultured *P. falciparum* gametocytes and fed to mosquitoes through an artificial membrane stretched across the base of a water-jacketed glass cylinder. The parasites in the blood meal were allowed to develop in the mosquito to the easily identifiable oocyst stage by maintaining the mosquitoes in a secured insectary for 6-8 days. Infectivity was measured by dissecting the midgut, staining it with mercurochrome, and then counting the number of oocysts per mosquito midgut of approximately 20 mosquitoes. The data was analyzed as described in Kaslow *et al* Vaccine Res. 2:95-103.

TABLE I  
3 WEEKS POST II VACCINATION

IMMUNOGEN DOSE		GEOM MEAN OOCYST (RANGE)		INFECTIVITY % OF CONTROL	<u>MOSO INFECTED</u> <u>MOSQ DISSECTED</u>
ALUM		5.3	(0-19)	100	22/23
Pfs 25	25 µg	2.2	(0-11)	42	18/21
	5 µg	7.3	(0-18)	138	22/23
25-28B	50 µg	0.52	(0-5)	10	11/24
	10 µg	0.49	(0-4)	9	9/23
25-28C	25 µg	0	(0-0)	0	0/22
	5 µg	0.3	(0-2)	5	7/23
25-28D	50 µg	2.8	(0-9)	53	19/20
	10 µg	4.7	(1-13)	89	23/23



TABLE II

**3 WEEKS POST III VACCINATION**

IMMUNOGEN DOSE		GEOM MEAN OOCYST (RANGE)		INFECTIVITY % OF CONTROL	<u>MOSO INFECTED</u> <u>MOSOQ DISSECTED</u>
ALUM		0.60	(0-5)	100	21/40
Pfs 25	25 µg	0.00	(0-0)	0	0/26
	5 µg	0.27	(0-3)	45	5/22
	1 µg	1.48	(0-8)	247	17/25
25-28B	50 µg	0.00	(0-0)	0	0/23
	10 µg	0.00	(0-0)	0	0/23
	2 µg	0.96	(0-5)	160	14/22
25-28C	25 µg	0.00	(0-0)	0	0/24
	5 µg	0.00	(0-0)	0	0/40
	1 µg	0.00	(0-0)	0	0/40
25-28D	50 µg	0.03	(0-1)	5	1/23
	10 µg	0.00	(0-0)	0	0/25
	2 µg	2.85	(0-16)	476	21/24

TABLE III

**4 MONTHS POST III VACCINATION**

IMMUNOGEN DOSE		GEOM MEAN OOCYST (RANGE)		INFECTIVITY % OF CONTROL	<u>MOSO INFECTED</u> <u>MOSQ DISSECTED</u>
ALUM		1.53	(0-5)	100	21/27
Pfs 25	25 µg	0.27	(0-3)	18	6/23
	5 µg	1.42	(0-7)	93	15/21
	1 µg	0.49	(0-3)	32	10/22
25-28B	50 µg	0.08	(0-1)	6	3/26
	10 µg	0.56	(0-4)	37	12/24
	2 µg	1.22	(0-5)	80	16/22
25-28C	25 µg	0.00	(0-0)	0	0/35
	5 µg	0.00	(0-0)	0	0/35
	1 µg	0.07	(0-1)	5	4/39
25-28D	50 µg	0.20	(0-1)	13	6/23
	10 µg	0.26	(0-2)	17	6/20
	2 µg	1.37	(0-10)	90	18/24

TABLE IV

**INFECTIVITY SUMMARY (% CONTROL)**

IMMUNOGEN DOSE		3 WEEKS POST II VACCINATION	6 WEEKS POST III VACCINATION	4 MONTHS POST III VACCINATION
ALUM		100	100	100
TBV 25	25 $\mu$ g	42	0	18
	5 $\mu$ g	138	45	93
	1 $\mu$ g	-	247	32
25-28B	50 $\mu$ g	10	0	6
	10 $\mu$ g	9	0	37
	2 $\mu$ g	-	160	80
25-28C	25 $\mu$ g	0	0	0
	5 $\mu$ g	5	0	0
	1 $\mu$ g	-	0	5
25-28D	50 $\mu$ g	53	5	13
	10 $\mu$ g	89	0	17
	2 $\mu$ g	-	476	90

All publications and patent applications cited in this specification are herein incorporated by reference for all purposes as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 804 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 60..716

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATAGATATGT ACATTATTAA TAATCACTTA TTATTTTATA TTTTITAGTT TTTTATACA  
59

ATG AAT ACA TAT TTT AAG GTA CTT CTT TTT TTA TTT ATT CAA CTT TAC  
107

Met Asn Thr Tyr Phe Lys Val Leu Leu Phe Leu Phe Ile Gln Leu Tyr  
1 5 10 15

ATA ACG TTG AAT AAG GCT CGG GTT ACT GAA AAT ACA ATA TGT AAA TAT  
155

Ile Thr Leu Asn Lys Ala Arg Val Thr Glu Asn Thr Ile Cys Lys Tyr  
20 25 30

GGT TAT TTA ATT CAG ATG AGT AAT CAT TAT GAA TGT AAG TGT ATT GAA  
203

Gly Tyr Leu Ile Gln Met Ser Asn His Tyr Glu Cys Lys Cys Ile Glu  
35 40 45

GGA TAT GTA TTA ATA AAT GAG GAC ACG TGT GGA AAA AAA GTA GTC TGT  
251

Gly Tyr Val Leu Ile Asn Glu Asp Thr Cys Gly Lys Lys Val Val Cys  
50 55 60

GAT AAA GTT GAA AAT TCA TTT AAA GCT TGT GAT GAA TAC GCT TAC TGT  
299

Asp Lys Val Glu Asn Ser Phe Lys Ala Cys Asp Glu Tyr Ala Tyr Cys  
65 70 75 80

TTC GAT TTA GGA AAT AAG AAT AAT GAA AAA CAG ATA AAA TGT ATG TGC  
347

Phe Asp Leu Gly Asn Lys Asn Asn Glu Lys Gln Ile Lys Cys Met Cys  
85 90 95

AGA ACA GAA TAT ACT TTA ACT GCT GGA GTA TGT GTT CCT AAT GTT TGT  
395

Arg Thr Glu Tyr Thr Leu Thr Ala Gly Val Cys Val Pro Asn Val Cys  
100 105 110

CGA GAT AAA GTA TGT GGT AAA GGA AAA TGT ATA GTA GAT CCT GCA AAT  
443  
Arg Asp Lys Val Cys Gly Lys Gly Lys Cys Ile Val Asp Pro Ala Asn  
115 120 125

TCT TTA ACA CAT ACA TGC TCA TGC AAT ATA GGT ACC ATA CTT AAC CAG  
491  
Ser Leu Thr His Thr Cys Ser Cys Asn Ile Gly Thr Ile Leu Asn Gln  
130 135 140

AAT AAA TTA TGT GAT ATA CAA GGT GAT ACA CCA TGT TCA TTA AAA TGT  
539  
Asn Lys Leu Cys Asp Ile Gln Gly Asp Thr Pro Cys Ser Leu Lys Cys  
145 150 155 160

GCA GAA AAT GAA GTG TGT ACA TTA GAA GGA AAT TAT TAT ACA TGT AAA  
587  
Ala Glu Asn Glu Val Cys Thr Leu Glu Gly Asn Tyr Tyr Thr Cys Lys  
165 170 175

GAA GAT CCT TCA TCT AAC GGA GGA GGA AAT ACT GTG GAC CAG GCT GAT  
635  
Glu Asp Pro Ser Ser Asn Gly Gly Gly Asn Thr Val Asp Gln Ala Asp  
180 185 190

ACA TCA TAT AGT GTA ATA AAC GGA GTA ACC CTA ACA CAC GTT CTG ATT  
683  
Thr Ser Tyr Ser Val Ile Asn Gly Val Thr Leu Thr His Val Leu Ile  
195 200 205

GTA TGC TCA ATA TTT ATT AAA TTG TTA ATA TAAAAAAAAA ATATATATAT  
733  
Val Cys Ser Ile Phe Ile Lys Leu Leu Ile  
210 215

ATGTATATAT ATATATATAT ATATATATAT ATATATATAT ATATATATGT CATATGATTT  
793

GCATCTTATT T  
804

## (2) INFORMATION FOR SEQ ID NO:2.

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 218 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Asn Thr Tyr Phe Lys Val Leu Leu Phe Leu Phe Ile Gln Leu Tyr
 1             5             10             15

Ile Thr Leu Asn Lys Ala Arg Val Thr Glu Asn Thr Ile Cys Lys Tyr
          20             25             30

Gly Tyr Leu Ile Gln Met Ser Asn His Tyr Glu Cys Lys Cys Ile Glu
          35             40             45

Gly Tyr Val Leu Ile Asn Glu Asp Thr Cys Gly Lys Lys Val Val Cys
          50             55             60

Asp Lys Val Glu Asn Ser Phe Lys Ala Cys Asp Glu Tyr Ala Tyr Cys
          65             70             75             80

Phe Asp Leu Gly Asn Lys Asn Asn Glu Lys Gln Ile Lys Cys Met Cys
          85             90             95

Arg Thr Glu Tyr Thr Leu Thr Ala Gly Val Cys Val Pro Asn Val Cys
          100            105            110

Arg Asp Lys Val Cys Gly Lys Gly Lys Cys Ile Val Asp Pro Ala Asn
          115            120            125

Ser Leu Thr His Thr Cys Ser Cys Asn Ile Gly Thr Ile Leu Asn Gln
          130            135            140

Asn Lys Leu Cys Asp Ile Gln Gly Asp Thr Pro Cys Ser Leu Lys Cys
          145            150            155            160

Ala Glu Asn Glu Val Cys Thr Leu Glu Gly Asn Tyr Tyr Thr Cys Lys
          165            170            175

Glu Asp Pro Ser Ser Asn Gly Gly Gly Asn Thr Val Asp Gln Ala Asp
          180            185            190

Thr Ser Tyr Ser Val Ile Asn Gly Val Thr Leu Thr His Val Leu Ile
          195            200            205

Val Cys Ser Ile Phe Ile Lys Leu Leu Ile
          210            215

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WHAT IS CLAIMED IS:

1                   1.     An immunogenic composition capable of eliciting an immunogenic  
2     response directed to an epitope comprising an isolated Pfs28 polypeptide and an isolated  
3     molecule comprising the epitope.

                  2.     The immunogenic composition of claim 1, wherein the isolated  
molecule comprising the epitope is a polysaccharide.

1                   3.     The immunogenic composition of claim 1, wherein the isolated  
2     molecule comprising the epitope is a polypeptide.

3                   4.     The immunogenic composition of claim 3, wherein the isolated  
4     molecule comprising the epitope is chemically linked to the Pfs28 polypeptide.

5                   5.     The immunogenic composition of claim 4, wherein the  
6     immunogenic composition comprises a Pfs28 fusion protein, wherein the Pfs28  
7     polypeptide is chemically linked to the isolated molecule comprising the epitope by a  
8     peptide bond.

1                   6.     The Pfs28 fusion protein of claim 5, wherein the fusion protein  
2     comprises a C terminal Pfs28 domain.

1                   7.     The Pfs28 fusion protein of claim 5, wherein the fusion protein  
2     comprises an N terminal Pfs28 domain.

                  8.     The Pfs28 fusion protein of claim 5, wherein the fusion protein  
comprises a Pfs25 domain.

1                   9.     The Pfs28 fusion protein of claim 5, wherein the fusion protein has  
2     a sequence selected from the group of amino acid sequences consisting of TBV25-Pfs28B,  
3     TBV25-Pfs28C, TBV25-Pfs28D, and conservative modifications thereof.

1                   10.     The Pfs28 fusion protein of claim 6, wherein the C terminal Pfs28  
2     domain comprises a carboxyl region of Pfs28.

1                   11.     The Pfs28 fusion protein of claim 5, wherein the fusion protein  
2     further comprises a flexible chemical linker.

1                   12.     The Pfs28 fusion protein of claim 11, wherein the flexible chemical  
2     linker comprises the sequence GGGPGGG.

1                   13.     The Pfs28 fusion protein of claim 5, wherein the fusion protein is a  
2     recombinant polypeptide.

1                   14.     An immunogenic composition comprising a pharmaceutically  
2     acceptable carrier and the Pfs28 fusion protein of claim 5.

1                   15.     The immunogenic composition of claim 14, wherein the  
2     immunogenic composition further comprises an adjuvant.

1                   16.     The immunogenic composition of claim 15, wherein the  
2     composition further comprises alum.

1                   17.     The immunogenic composition of claim 14, wherein the fusion  
2     protein, when administered to a mammal, elicits a transmission blocking immune response.

3                   18.     A nucleic acid encoding the fusion protein of claim 5.

1                   19.     The nucleic acid of claim 19, wherein the fusion protein has a  
2     sequence selected from the group of amino acid sequences consisting of TBV25-Pfs28B,  
3     TBV25-Pfs28C, TBV25-Pfs28D, and conservative modifications thereof.



1                   20.     The nucleic acid of claim 19, wherein the nucleic acid is selected  
2     from the group of nucleic acids consisting of TBV25-Pfs28B, TBV25-Pfs28C,  
3     TBV25-Pfs28D, and conservative modifications thereof.

4                   21.     The nucleic acid of claim 19, wherein the nucleic acid hybridizes  
5     under stringent conditions to a nucleic acid selected from the group consisting of  
6     TBV25-Pfs28B, TBV25-Pfs28C, and TBV25-Pfs28D.

1                   22.     The nucleic acid of claim 19, wherein the nucleic acid comprises  
2     yeast preferred codons which enhance translation of the nucleic acid in yeast.

1                   23.     The nucleic acid of claim 19, further comprising a pharmaceutical  
2     excipient.

1                   24.     The nucleic acid of claim 19, wherein protein encoded by the  
2     nucleic acid is secreted from a culture of yeast at a level in excess of 5 mg/L.

1                   25.     A vector comprising the nucleic acid of claim 19.

1                   26.     The vector of claim 26, wherein the vector is expressed in yeast.

2                   27.     The vector of claim 26, wherein the vector further comprises a  
3     promoter.

1                   28.     A cell comprising the nucleic acid of claim 19.

2                   29.     The cell of claim 29, wherein the cell is a yeast cell.

3                   30.     A method of inducing a transmission blocking immune response in a  
4     mammal, comprising administering the Pfs28 fusion protein of claim 5 to a mammal.

- 5                   **31.**    The method of claim 30, wherein the Pfs28 fusion protein  
6                   comprises a Pfs25 domain.
- 7                   **32.**    The method of claim 30, wherein the Pfs28 fusion protein is  
8                   administered intramuscularly, intradermally, or subcutaneously.
- 1                   **33.**    The method of claim 30, wherein the Pfs28 protein is administered  
2                   to the mammal with an adjuvant.
- 1                   **34.**    The method of claim 33, wherein the adjuvant is alum.
- 35.**    A method of eliciting an immunogenic response directed to an  
                  epitope comprising administering an isolated Pfs28 and an isolated molecule comprising  
                  the epitope.

# INTERNATIONAL SEARCH REPORT

onal Application No

PCT/US 97/17666

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/445 C12N15/81 A61K39/015

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GOZAR M.M. ET AL.,: "TBV-25-28 fusion protein is more potent than TBV35H as a plasmodium falciparum transmission-blocking vaccine" AM. J. TROP. MED. HYG. SUPPL., vol. 55, no. 2, - August 1996 pages 192-193, XP002057802 see the whole document ---	1-35
A	QUAKYI I. A. ET AL.,: "The 230-kDa gamete surface protein of plasmodium falciparum is also target for transmission-blocking antibodies" J. IMMUNOL., vol. 139, no. 12, - 15 December 1987 pages 4213-4217, XP002057803 cited in the application see the whole document --- -/-	1-35

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

5 March 1998

Date of mailing of the international search report

17.03.98

Name and mailing address of the ISA

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Authorized officer

Müller, F

# INTERNATIONAL SEARCH REPORT

Original Application No  
PCT/US 97/17666

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 01552 A (US ARMY) 20 January 1994 see the whole document ---	1-35
A	WO 92 14486 A (US) 3 September 1992 see whole document esp. claims and abstract ---	1-35
A	KASLOW D. C & SHILOACH J.: "Production, purification and immunogenicity of a malaria transmission-blocking vaccine candidate: TBV25H expressed in yeast and purified using nickle-NTA agarose" BIO/TECHNOLOGY, vol. 12, - May 1994 pages 494-499, XP002057804 cited in the application see esp. discussion ---	1-35
P,X	DUFFY P. E. & KASLOW D. C.: "A novel malaria protein, Pfs28, and Pfs25 are genetically linked and synergistic as falciparum malaria transmission-blocking vaccines" INFECTION AND IMMUNITY, vol. 65, no. 3, - March 1997 pages 1109-1113, XP002057805 see whole document, esp. discussion ---	1-35
T	GOZAR M.M. ET AL.,: "Saccharomyces cervisiae- secreted fusion proteins pfs25 and pfs28 elicit potent plasmodium falciparum transmission-blocking antibodies in mice" INFECTION AND IMMUNITY, vol. 66, no. 1, - January 1998 pages 59-64, XP002057806 see the whole document -----	

# INTERNATIONAL SEARCH REPORT

mational application No.  
PCT/US 97/17666

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 17, 30-35  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 17, 30-35 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9401552 A	20-01-94	AU 675445 B	06-02-97
		AU 4670493 A	31-01-94
		CA 2138646 A	20-01-94
		EP 0651798 A	10-05-95
		JP 8502402 T	19-03-96
		US 5527700 A	18-06-96
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WO 9214486 A	03-09-92	AU 1588092 A	15-09-92
		US 5217898 A	08-06-93
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